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THE CONTRACTILE VACUOLE IN *PARAMECIUM TRICHIUM.*

ROBERT L. KING,

ZOÖLOGICAL LABORATORY, UNIVERSITY OF PENNSYLVANIA.

INTRODUCTION.

The contractile vacuolar apparatus of Ciliata seems to be more highly specialized than that of other Protozoa. As to the form of the vacuole itself, we may recognize for convenience two general types among the Ciliata: (*a*) Vesicle-fed vacuoles, in which the contracting vacuole is surrounded by a series of small vacuoles (called vesicles in this paper) which seem to coalesce and form a new contracting vacuole after systole, new smaller vesicles appearing around the contracting vacuole after systole, and growing larger during diastole as described by Taylor ('23) in *Euplotes;* (*b*) Canal-fed vacuoles, in which the vacuole is fed by canals, which vary in number from one in *Spirostomum* and *Stentor* (Roux, '01) to thirty in *Ophryoglena* (Bütschli, '87–'89). A condition which seems to be intermediate has been described by Schewiakoff ('89) for *Prorodon teres.* Here the vacuole is fed by four radial rows of vesicles which decrease in size distally from the contractile vacuole.

The contractile vacuole usually communicates with the exterior by a tubule through a pore. This excretory tubule leading from the vacuole to the pore may be very short as in *Nassula* and *Frontonia* relatively long as in *Urocentrum,* or very long as in *Lembadion* (Schewiakoff, '89) where the vacuole is located near the central part of the body and the excretory tube leads to the pore which is about one quarter of the body length posterior to the vacuole.

The purpose of this paper is to describe the contractile vacuolar apparatus of *Paramecium trichium* which has been described by Stokes ('88) as consisting of two vacuoles "close together, contracting quickly, the one beginning to again form almost before the completion of the other's systole" and by Wenrich ('26) who was unable to reach a satisfactory conclusion as to "whether the two main vacuoles empty alternately into the same outlet tube or whether there is an auriculo-ventricular relationship between them."

Without the use of methods introduced by Bresslau ('21) as modified in this paper, the structures to be described could not have been studied. I am also particularly indebted to Doctor D. H. Wenrich who first pointed out the need for further study of the contractile vacuoles of *Paramecium trichium*.

Materials and Methods.

The *Paramecium trichium* used in this series of observations were obtained from two sources in the vicinity of Swarthmore, Pa.: from the east branch of Crum Lynne Creek where they were found among the jelly of toads eggs and from the west branch of Stony Creek, the source of Wenrich's ('26) Swarthmore race. The material was first studied in mixed cultures but later pure lines were established. For purposes of comparison, *Colpidium colpoda* from the same sources was studied.

Observations on the contractile vacuolar apparatus of *Paramecium trichium* were first made on material prepared according to the methods described by Bresslau ('21) and later those described by Coles ('27). These methods consist essentially of drying the protozoa in concentrated solutions of stains, which are relatively low in toxicity. The dye is precipitated in and on surface structures such as the depressions from which cilia arise, the cilia themselves, the mouth and the cytopharynx. The routine used is essentially as follows: A small drop of concentrated culture of the protozoa to be examined is placed upon a perfectly clean glass slip and mixed with a similarly sized drop of the stain. The two are then mixed and spread evenly over the slip with a needle or a glass spreader. The slides are then allowed to dry in the air and are examined under oil or mounted in damar.

Bresslau ('21) has used the following staining solutions: (*a*) Three parts saturated aqueous solution China Blue to one part saturated aqueous solution Cyanosin (a mixture previously used in bacteriological technique) and (*b*) one cc. 10 per cent. aqueous solution Opal Blue to 4–6 drops 6.5 per cent. aqueous solution Phloxinrhodamin.

Coles ('27) has used numerous stains for Protozoa in the same manner, obtaining his best results with a saturated aqueous solution Nigrosin.

The stains recommended by Bresslau are very toxic to Paramecium trichium which are killed quickly and usually burst open before they become dry. However, with solutions of (*a*) 10 per cent. China Blue (Coleman Bell), (*b*) 10 per cent. Nigrosin (Coleman Bell or Grübler), (*c*) Mixture of equal parts of above, and (*d*) 10 per cent. Opal Blue (Coleman Bell), beautiful preparations have been made. The above methods have been placed in order of their apparent usefulness for the structures studied in *Paramecium trichium.*

Observations made on such dried preparations have been verified on material fixed with Bouin's or Schaudinn's and stained with hæmalum and on living animals in hanging drops of the culture medium or of the culture medium mixed with China Blue, Opal Blue or Nigrosin. *Paramecium trichium* will survive over two hours in such hanging drops containing stains and remain apparently typical during that time.

Observations.

There are two contractile vacuoles in *Paramecium trichium,* one located in the anterior and one in the posterior part of the animal (Wenrich, '26). When dried in the staining solutions described above the coloring matter collects in the contractile vacuolar apparatus sometimes penetrating into the vacuole itself. The contractile vacuole is seen to be connected with the exterior by a long convoluted tubule (Figs. 2, 3, 4 and 9) which terminates in a small pore (Figs. 1, 2 and 4) located between the longitudinal rows of cilia on the dorsal surface of the body opposite the mouth. The pore of the anterior vacuole and that of the posterior open to the exterior between the same or adjacent rows of longitudinal

rows of cilia (Figs. 8, 15 and 14). The vacuoles themselves do not seem to be fixed in position in living animals but move about more or less in the endoplasm; this apparently is made possible by the presence of the convoluted tubule. The inner end of the tubule terminates in a cup-like valve (Figs. 7 and 9) with which the vacuole is in contact when undergoing systole. This cup-like valve has been observed both in prepared slides and in living animals; while it seems always to be in contact with the vacuole it may be exterior or interior, anterior or posterior in relation to the vacuole. In fact the whole structure seems to be continually in active movement. The proximal portion of the tubule shows the effect of increased pressure at the beginning of systole, tending to straighten out as does a coiled hose when water is first turned into it under pressure.

In living animals each of the contractile vacuoles appears to be made up of two vacuoles which contract alternately. However, if they be carefully compared with a typical vesicle-fed vacuole such as that of *Colpidium colpoda,* it is found that the two are fundamentally alike. Observations made with a stop-watch show that the time relations of various parts of the cycle of systole and diastole in the two species are quite different. The table gives a set of typical observations on *Paramecium trichium* and *Colpidium colpoda.* These observations were made upon a single individual of each species on the same slide. For the interval between contractions seven groups of three contractions each were timed in *Colpidium colpoda;* ten groups of three contractions each in *Paramecium trichium* (anterior vacuole). A number of separate observations of the time from beginning to end of systole were made on the same individuals.

TABLE I.

TIME RELATIONS IN SECONDS OF CONTRACTILE VACUOLES OF *Paramecium trichium* AND *Colpidium colpoda* AT 25° C.

	Time between Completion of Two Systoles.	Time for Systole.
Paramecium trichium	3.1	2.0
Colpidium colpoda	7.4	0.4

In *Colpidium colpoda* the vacuole empties rapidly (about 0.4 seconds); the small feeding vesicles gradually enlarge, coalesce

into one vacuole which finally reaches its maximum size about seven seconds after the last contraction, in the meantime there have appeared more tiny feeding vesicles around its periphery. The vacuole then contracts rapidly and the cycle is repeated.

In *Paramecium trichium* the vacuole (Fig. 12*a*) empties slowly (about 2 seconds). As it contracts the feeding vesicles grow (Fig. 12*b*) and by the time the vacuole has half completed systole (Fig. 12*c*) the feeding vacuoles coalesce with an apparent passing of membranes over their surface marking their fusion [called by Taylor ('23) "vestiges of the adjacent walls" in *Euplotes*]. When systole is completed (Fig. 12*d*) the new contractile vacuole has reached its maximum size; as it comes in contact with the cup-like valve a membrane (as described by Wenrich, '26) appears to pass over its surface, and it begins to contract (Fig. 12*e*).

The two processes are fundamentally alike; in *Paramecium trichium* the systole of the old contractile vacuole and the diastole of the new occur almost simultaneously and are of approximately the same duration while in *Colpidium colpoda* they occur consecutively with systole very brief and diastole protracted.

Summary.

1. There is an anterior and a posterior contractile vacuolar apparatus in *Paramecium trichium*.

2. Each is permanent and consists of feeding vesicles, contractile vacuole, excretory tube and pore.

3. The contractile vacuoles of *Paramecium trichium* are vesicle-fed, differing here from those of other well known species in the same genus which are canal-fed.

4. Diastole of the new vacuole is practically complete before the prolonged systole of the old is over. This gives the appearance of two vacuoles contracting alternately.

5. The excretory tube is long and convoluted with its flattened cup-like end in contact with the contractile vacuole.

6. The excretory tube opens as an excretory pore on the surface of the body opposite the mouth.

7. The pore of the anterior and that of the posterior apparatus are located between the same or adjacent longitudinal rows of cilia.

8. The long duration of systole may be correlated with the presence of the convoluted excretory tube.

9. The excretory tube and pore were first demonstrated by the use of Bresslau's relief staining method.

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PLATE I.

Explanation of Figures.

Figs. 1–11. Contractile vacuolar apparatus of *Paramecium trichium*, dried in 10 per cent. China Blue unless otherwise stated × 1000.

1. Posterior apparatus of animal photographed for Fig. 13.
2. Anterior apparatus of animal photographed for Fig. 14.
3. Posterior apparatus.
4. Posterior apparatus.
5. Anterior apparatus.
6. Posterior apparatus of same animal as Fig. 5.
7. Posterior apparatus of animal photographed for Fig. 16.
8. Entire animal showing cytopharynx, anterior and posterior vacuolar apparatus. Same as photograph Fig. 15.
9. Anterior apparatus.
10. Anterior apparatus (10 per cent. nigrosin).
11. Two tubules and pores from anterior end of same animal.

Fig. 12. Diagrams of various stages of contracting vacuole. *a*. Beginning of systole. *b*. Systole half-completed, feeding vesicles grow larger. *c*. Fusion of feeding vesicles. *d*. Systole complete. Cup-like inner end of tubule. *e*. Fusion of new contractile vacuole with cup-like inner end of tubule preparatory to systole.

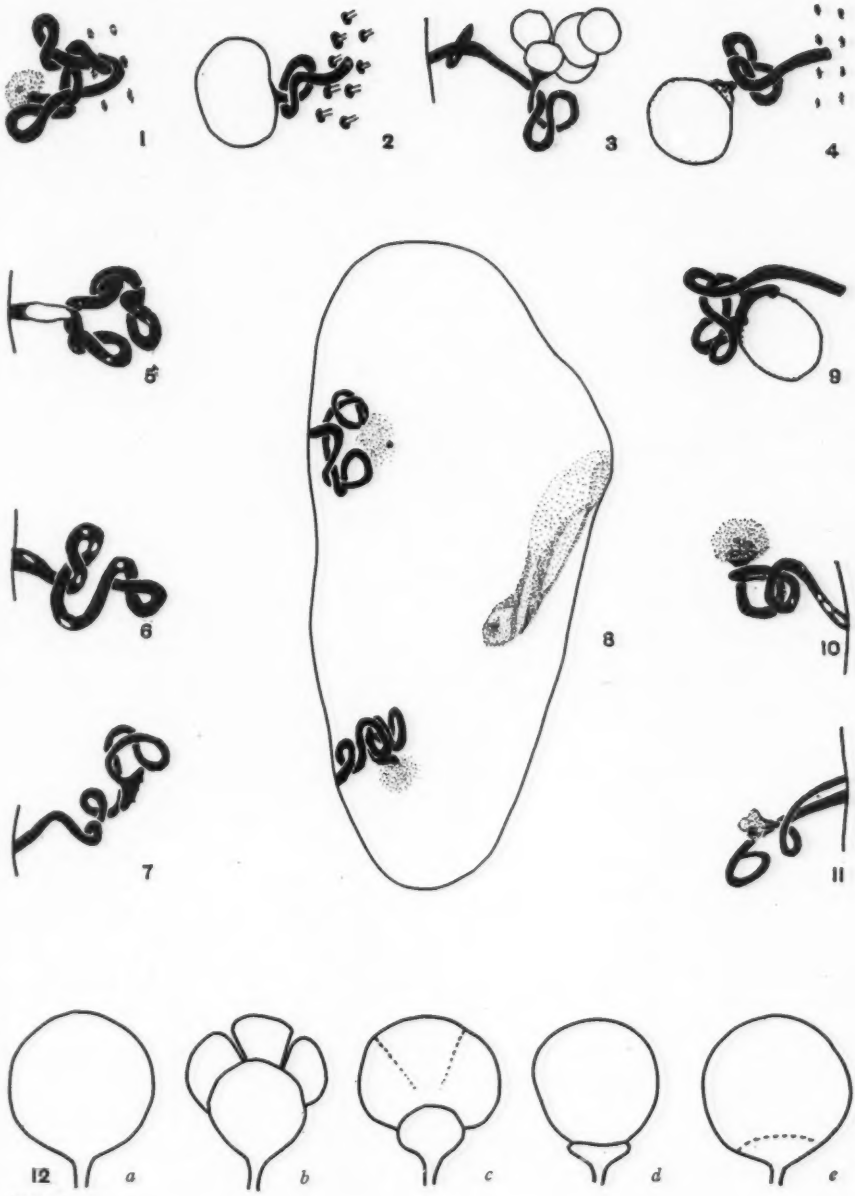

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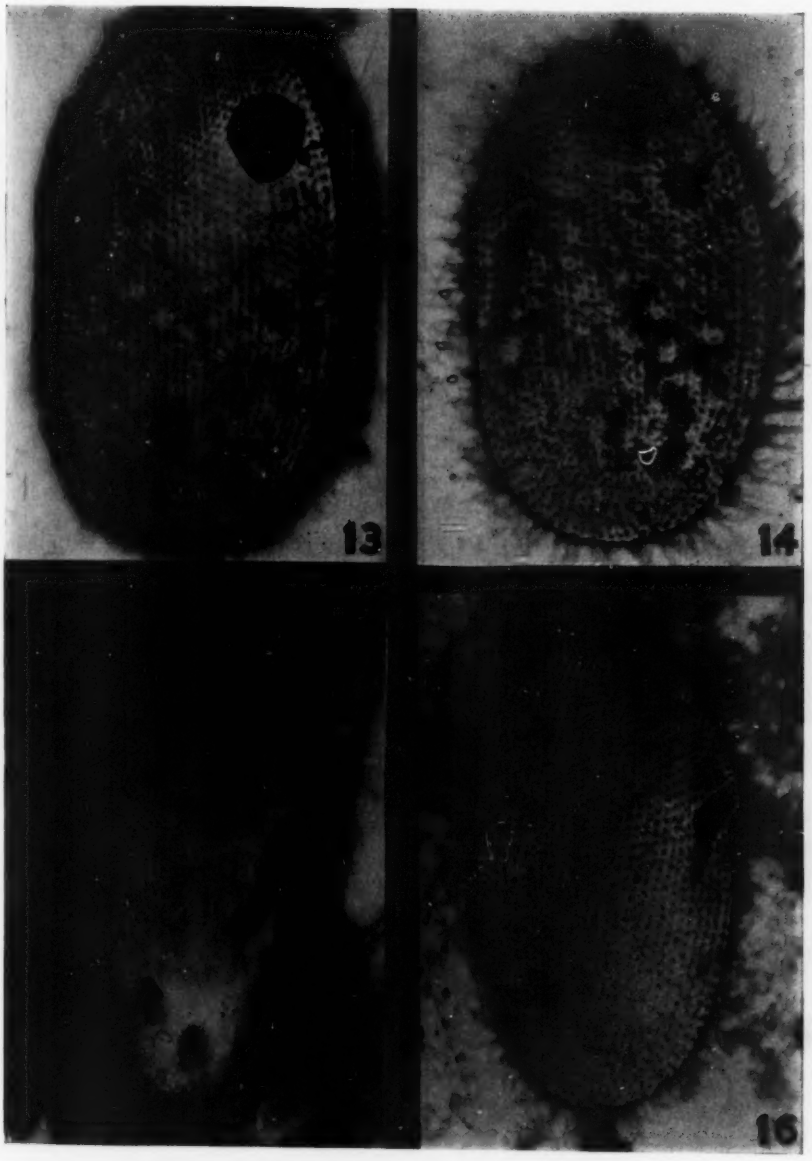
PLATE II.

Photographs × 750.



ROBERT L. KING.

OBSERVATIONS OF THE FEEDING MECHANISM OF A CTENOPHORE, *MNEMIOPSIS LEIDYI*.[1]

ROLLAND J. MAIN.

Observations of the feeding habits of ctenophores are scattered through the literature dealing with these organisms (Bigelow, '15; Lebour, '22–'23; Mayer, '12; Nelson, '25), but as yet no detailed study of the feeding mechanism has appeared.

The ciliation of a hydromedusa has been studied (J. F. Gemmill, '19), but this compares in no way with the complex food catching apparatus of a ctenophore such as *Mnemiopsis leidyi*. The morphological work done upon this ctenophore is incomplete, for neither Agassiz (1849), Fewkes (1881), nor Mayer ('12), mention the presence of its remarkable mechanism for the capture of food.

Mnemiopsis leidyi through its habit of devouring the free-swimming larvæ of the oyster and of other molluscs becomes of such economic importance that it is of interest to determine by what means these organisms are captured and carried into the stomodæum, and how the undigested residues are discharged.[2]

Materials and Methods.

The specimens of *Mnemiopsis leidyi* were obtained in the northern half of Barnegat Bay, N. J., a shallow estuary, in water of a specific gravity approximating 1.010, with temperatures close to 20° C., during August and the first part of September, 1926. The animals were caught in a net, placed in jars without injury and within ten minutes after capture they were being examined under the binocular.

Living plankton was used to determine the feeding mechanism, and it is felt that to this the success of the experiment is pri-

[1] From the Zoölogical Laboratory of Rutgers University, Publication No. 11, New Jersey Oyster Investigation Laboratory.

[2] The writer is indebted to Dr. Thurlow C. Nelson of Rutgers University for aid and advice during this investigation and for reading the manuscript.

marily due, since it is doubtful for reasons given below whether any other material could have been successfully used. The plankton was secured by pouring sea water through a 200 mesh plankton net, and concentrating the organisms in a small amount of water. A *Mnemiopsis* was placed in a watch crystal under the binocular, a little of the plankton culture was added, and the reactions of the ctenophore noted.

Structure and Operation of the Food Catching Mechanism.

To understand the mechanism of the food catching apparatus, it is first necessary to have a clear idea of the gross anatomy of *Mnemiopsis,* Fig. 1. Although considerable work has been done

Fig. 1. Adult *Mnemiopsis leidyi* from Barnegat Bay. Photographed immediately after fixation in 10 per cent. hydrochloric acid. The oral lobes have contracted to approximately 2/3 the length characteristic of the living animal. Photographed by T. C. Nelson.

on the morphology of the animal, all the writers have apparently disregarded the presence of a definite ridge, an extension of the

lips of the mouth, which the writer has named the "labial ridge."[8]

There are four furrows formed by the juncture of the oral lobes with the body. In each furrow along the inner side of the labial ridge is a line of tentacles. Through the base of this labial ridge runs a branch of the paragastric canal, which finally unites with the auricular canal. On the opposite side of this ridge is the ciliated channel for conveying food to the mouth, Figs. 2 and 3.

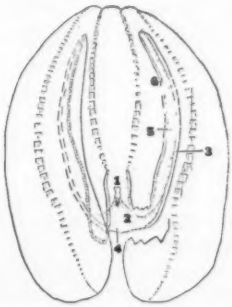


FIG. 2. *Adult Mnemiopsis leidyi.* Part of the right lobe and the tip of the right auricle have been omitted. It is difficult to represent the turning of the labial ridge. The lips are in the plane of the paper. As the lip becomes the labial ridge, it turns so that it lies in a plane at right angles to the paper. 1. The tentacular bulb. 2. The tentacular ridge, with tentacles. 3. The labial ridge, along which runs the tentacular ridge. 4. Lip. 5. Auricular groove. 6. Cilia of auricle.

To this channel, or trough, will be applied the term "labial trough." It is formed by the labial ridge on one side, and the oral lobe on the other. It runs along the ridge to the point where the ridge becomes the lip, and here the trough runs directly into the corner of the mouth, Fig. 4. The labial ridge is separated from the cilia of the auricles by the auricular groove in which the cilia of the auricle beat, and at the bottom of which lie the tentacles stretched out in the current.

[8] The writer calls attention to some apparent discrepancies in earlier work on *Mnemiopsis leidyi.* Fewkes pictures an adult of this species which differs widely from the type obtained from Barnegat Bay. The latter, save for the contraction of the oral lobes, is well illustrated in Figure I. Fewkes' figure shows the surface of the animal covered with discoidal warts which are claimed by Mayer to be present in *M. mccradyi* and in *M. gardeni* but absent in *M. leidyi.* Fewkes' figure differs also in the shape of the body.

Near the mouth the line of tentacles curves away from the labial ridge up to the tentacular bulb. The tentacles are placed irregularly along this line, usually in groups, some animals having

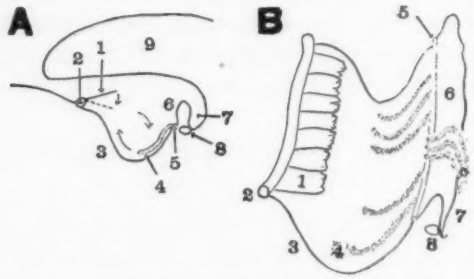


FIG. 3. *A*. Cross section of auricular groove. The cilia of the auricle (1) beat up and down as indicated by the arrow and dotted line. The other two arrows show the direction of the current produced by the cilia. 1. Cilia of auricle. 2. Auricular canal. 3. Auricular groove. 4. Tentacle. 5. Tentacular ridge. 6. Labial ridge. 7. Labial trough. 8. Branch of paragastric canal. 9. Oral lobe. *B*. View of auricular groove from above. The oral lobe has been laid back. Parts correspond to Fig. 3*A*. Three tentacles are here shown putting food in the labial trough, where it will be drawn off and conveyed to the mouth.

many more tentacles than others. This may be due to the fact that they have been broken off in securing food, for often food may be seen entering the stomodæum with portions of tentacles attached.

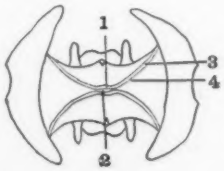


FIG. 4. Oral view of adult *Mnemiopsis leidyi*. This shows how the lips are continued into the labial ridge and how the trough runs into the mouth. 1. Lip. 2. Mouth. 3. Labial ridge. 4. Labial trough.

When a particle of food is caught in the current produced by the cilia in the grooves it is whirled about until it finally touches a tentacle. This entangles it, often with the aid of several other tentacles. These tentacles then contract, and

apparently are drawn over the labial ridge into the labial trough, presumably by cilia, Fig. 3*B*. Here they stretch out in the direction of the mouth, the food is drawn off, and passes down toward the mouth. The tentacles then relax, and resume their normal position. Often several pieces of food are beaten about for some time in the groove. Dirt in the groove is gradually entangled in mucus into a long thread which slowly passes out at the aboral end of the groove. If much dirt be present, the whole animal pulsates, contracting the groove and forcing out all material present. The tentacles were never seen placing any foreign material into the labial trough, unless a little happened to be caught up with the food. Possibly it is for these reasons that *Mnemiopsis leidyi* is not found in muddy waters, since it will not seize food if much dirt be present. Carmine introduced directly into the labial trough is drawn along but for a short distance, and then is passed out over the labial ridge. For this reason the use of the natural plankton food organisms in studying the mechanism is imperative.

It is here that we must search for the explanation of why *Mnemiopsis leidyi* lives so largely upon bivalve larvæ, in spite of the great preponderance of other plankton in the water (Nelson, '25). The writer has observed that often the ctenophore is unable to hold an active copepod. Possibly the stronger swimmers are able to escape the ciliary currents, whereas the young oyster shuts its shell on contact and is therefore an easy prey. Polychæte larvæ were found in *Mnemiopsis* at this time, although never more than one or two per animal. This is contrary to Nelson's ('25) belief that it would be almost impossible for this ctenophore to ingest such a prey.

Food captured by the tentacles about the mouth was passed down directly over the lips into the mouth, often aided by a contraction of the lips, bringing them near the tentacular bulb. After the food has passed into the stomodæum, it usually proceeds slowly to the center, between the two paragastric canals, close to the convoluted tubules which probably secrete the digestive juices. It may, however, lodge below this point, Fig. 5. Sometimes it is caught in the swifter current at the very edge of the stomodæum, and is whirled up to the beating cilia at the aboral end. Here it

is usually turned back, for these cilia seem to act partly as filters. At times, however, a particle may be squeezed through and enter the funnel to pass around in the food canals.

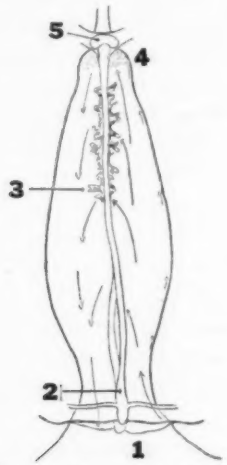


FIG. 5. The stomodæum of *Mnemiopsis leidyi*. In order to avoid confusion, the paths taken by ingested food are shown on the right side only. The larger arrows are the more usual paths. The smaller arrows on the extreme right denote a swifter current, in which the food sometimes travels. On the left half only, are shown the paths taken by the excreted materials. 1. Mouth. 2. Paragastric canal. 3. Digestive glands? 4. Cilia. 5. Funnel.

The undigested material in the stomodæum is passed down as indicated, and ejected through the mouth. These paths are not definite, for incoming food will pass a certain spot, and immediately afterwards outgoing wastes will cross the same spot going in the opposite direction. Those particles which have passed through into the food canals may reënter the stomodæum and pass out through the mouth, or they may follow the usual procedure for material in the canals, and be voided at the anus.

Just before defecation occurs, particles may be seen gathering about in the funnel and in the axial funnel canal. Then one of the branches of this canal elongates above the surface and the particles are forced out through the pore. The current in all the food canals now seems to be in the direction of the funnel. By

this time the cilia of the aboral end of the stomodæum have ceased beating, and the whole upper end of the stomodæum presents a contracted appearance, Fig. 6. After the particles of waste have

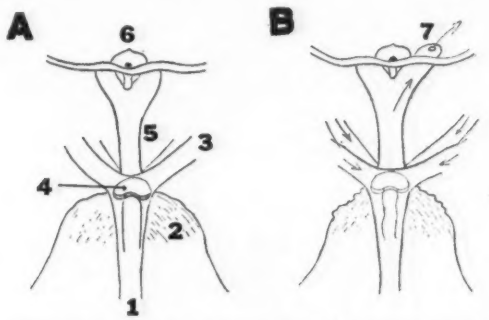


FIG. 6. Aboral portion of stomodæum, and axial funnel canal of *Mnemiopsis leidyi*. *A*. Before defecation. 1. Paragastric canal. 2. Cilia. 3. Food canals. 4. Funnel. 5. Axial funnel canal. 6. Sense organ. 7. Excretory pore. *B*. During defecation, arrows showing direction of waste. Note shrunken appearance of stomodæum.

all passed out the cilia begin beating again, and the branch of the funnel canal slowly retracts. Although several successive defecations of specimens have been observed, only one branch was used, and in no specimen was the use of both branches observed.

THE EARLY DEVELOPMENT OF THE FOOD CATCHING MECHANISM.

Since the complex food catching apparatus is present only in the adult *Mnemiopsis*, the question of its ontogeny naturally arises. The young were plentiful at the time of this study, and various stages were examined.

The smallest specimens obtained were in the Cydippidæ-stage, approximately 2 mm. high and 2 mm. broad, Fig. 7. These possess two long branching tentacles with no trace of the tentacular ridge, labial ridge, or labial trough. They feed by capturing the food with the tentacles, retracting them, and drawing them down over the lip and into the stomodæum, where the food is drawn off. Another contraction, and the tentacles emerge, to again float up above the animal.

The next step in development was found in a 6 mm. specimen, Fig. 8. This stage has still the two compound tentacles.

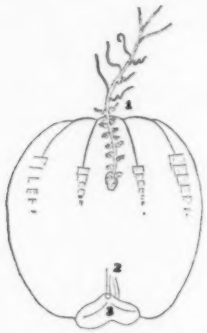


FIG. 7. Young *Mnemiopsis leidyi*, 2 mm. high. 1. Branching tentacle, partially contracted. 2. Paragastric canals, only unbranched terminations shown. 3. Mouth.

The 8 mm. specimens are much further advanced, Fig. 9. The auricles are now forming, and the tentacular ridge has appeared as a slight fold or line as shown, but it is not connected to the tentacular bulb, and possesses no tentacles. It was observed that tentacles never appeared along the tentacular ridge until it had joined the tentacular bulb.

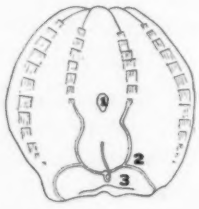


FIG. 8. Young *Mnemiopsis leidyi*, 6 mm. high. 1. Tentacular bulb. (Tentacle omitted, being same as in Fig. 7.) 2. Juncture of paragastric and auricular canals. 3. Mouth.

It is now easy to see how the adult structures are completed. As the junction of the paragastric and auricular canals moves upward forming the auricular groove, the tentacular ridge and labial ridge grow with it. The large branched tentacle disappears, and small tentacles appear along the tentacular ridge.

This remarkable food catching apparatus of *Mnemiopsis,* in which the conveying system seems to foreshadow that of the bivalves, is certainly a great advance over that of the Scyphozoa.

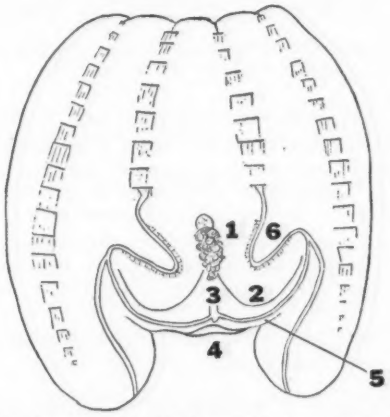


FIG. 9. Young *Mnemiopsis leidyi,* 8 mm. high. 1. Branched tentacle entirely retracted, but same as in Fig. 7. 2. Tentacular ridge. 3. Paragastric canal, termination shown with branches. 4. Mouth. 5. Beginning of labial ridge. 6. Developing auricles.

Of its efficiency there can be no doubt, for compare Bigelow's ('15) statement that the plankton was greatly diminished in a swarm of ctenophores. Nelson ('25) also brings forth evidence of a correlation between the abundance of *Mnemiopsis leidyi* and the intensity of shipworm infestation and oyster sets. Moreover, the fact that the ctenophores are usually found in such vast and dense swarms, argues well for their ability to obtain food. Possibly it is due to this efficient apparatus that we find in many species of ctenophores the small compact bodies and absence of long trailing tentacles.

SUMMARY.

The mode of feeding was studied in young tentacled forms and in the adult *Mnemiopsis leidyi.* The young capture food with their branched tentacles, and deposit it in the mouth. The adults entangle the food with the small tentacles along the tentacular ridge, and deposit it in the labial trough, whence it is carried to the mouth.

Food enters the stomodæum and after digestion is cast out of the mouth, or it may enter the food canals and pass out of the anus.

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THE INFLUENCE OF OXYGEN TENSION UPON THE RESPIRATION OF UNICELLULAR ORGANISMS.

WILLIAM R. AMBERSON.

(From the Department of Physiology, School of Medicine, University of Pennsylvania, and the Marine Biological Laboratory, Woods Hole, Mass.)

Our knowledge of the influence of oxygen tension upon the oxygen consumption of unicellular organisms is quite incomplete. The literature contains many studies of the influence of oxygen tension changes upon growth and activity of such forms, but relatively few direct measurements of oxygen consumption have been made. In some studies in which the consumption has been measured the problem has been complicated by changes in the number of respiring cells during the course of the experiment. This would appear to be true of such observations as those of Stephenson and Whetham (1924) who have found that the oxygen intake of *B. coli* is much greater in pure oxygen than in air, and of Novy and Soule (1925) who report that the tubercle bacillus grows best in an atmosphere containing 40–50 per cent. oxygen, the growth and the oxygen consumption falling off progressively above and below this value. It is not possible to infer that a change in division rate indicates a change in the oxygen intake of the individual bacterium. The influence of the oxygen tension may be more indirect, possibly through the formation of such growth-promoting substances as Burrows (1924) has described, whose production is increased by an increased oxygen supply.

In other studies of bacterial respiration in which there has probably been no significant change in the number of respiring cells, Pütter (1924) and E. N. Harvey (1926) have secured evidence that the respiratory rate is not influenced by changes in the oxygen tension. In unicellular animal organisms the weight of the somewhat meagre evidence so far secured indicates that oxygen consumption is independent of oxygen tension over a wide range. Lund (1918) found this to be true for *Paramecium*. Henze (1910) and Warburg (1908) found a similar situation in

sea-urchin eggs, in which there was little change in oxygen intake when the oxygen tension varied from double that in air to one-fourth of the same value.

In all of the studies in this last group in which oxygen has actually been measured, the Winkler method has been employed. It is well known that this method, while very satisfactory for the determination of dissolved oxygen in pure water or in salt solutions, becomes untrustworthy when organic material is present in the fluids tested. Heilbrunn (1915) and others have objected to the use of the method in the study of heavy suspensions of protozoa and marine eggs. The presence of iron, found by Warburg (1914) to be contained in sea-urchin eggs in considerable amounts, is known to introduce large errors in the titration. (See Alsterberg, 1926.)

I became interested in this problem after making the observation (1924) that the oxygen consumption of a number of marine invertebrates is directly proportional to the oxygen tension in the sea water, over a considerable part of the normal physiological range. This observation has led me to a reëxamination of the problem in other forms. The present communication deals with some results obtained on unicellular materials in an attempt to confirm the conclusions of previous workers by methods not open to the criticisms which can be leveled against the Winkler technique. This confirmation has been secured. The data are submitted in support of the older observations, and as giving a more complete account of the oxygen tension relationships in the *Arbacia* egg than has previously been published.

On the technical side an attempt has been made to apply standard methods of gas analysis to the study of the problem. Novy and his collaborators have previously successfully used such methods in their study of bacterial respiration. I find that the oxygen consumption of unicellular animal organisms can be similarly followed by such methods, with an accuracy at least as good as that possible in human and mammalian metabolic studies. The carbon dioxide production is more difficult to determine because of the high solubility of the gas in the liquid phase, and the possibility of its chemical fixation. No great reliance can therefore be placed upon the carbon dioxide values given below, or upon the

respiratory quotients calculated. The large variations in the value of the quotient is sufficient to indicate the magnitude of the errors which must be present in the determination of carbon dioxide. My main concern has been to study the oxygen consumption.

Experiments with *Paramecium*.

A group of experiments was first carried out with *Paramecium*, in an attempt to develop a satisfactory technique. For several reasons the data obtained are not as complete or accurate as the values secured later on *Arbacia* eggs. The results are, however, fairly consistent and give a satisfactory confirmation of Lund's report on this organism.

A thick suspension of the protozoa was prepared by centrifuging several liters of fluid from a number of cultures. The organisms were then washed through several changes of tap water, being concentrated with the centrifuge after each washing. The suspension in its final form was practically free from bacteria. The cultures were never entirely pure, but *P. caudatum* always constituted at least 95 per cent. of the protozoa present. The presence of other unicellular organisms, either animal or plant, cannot appreciably have modified the results.

A preliminary obstacle was encountered when it was observed that it is exceedingly difficult to secure two samples of such a suspension which will contain the same number of animals. This difficulty arises from the high mobility of the organisms which are negatively geotropic, and tend to rise to the surface even while the sample is being drawn. After many unsuccessful attempts to secure two identical samples, the procedure was abandoned. In its stead it was found possible to carry out two consecutive measurements of respiration upon the same suspension, the first at atmospheric pressure, the second at some lower or higher pressure. Under the conditions of the experiments division was absent, yet the measurements were completed before starvation intervened.

20 cc. of the suspension finally obtained were introduced into a cylindrical glass vessel, of about the size and form of a Haldane gas collecting tube. This tube was fitted with three-way stopcocks at both ends. The volume was 80.85 cc. After the introduction of the suspension the volume of gas in the tube was, therefore,

60.85 cc. Air delivered by a pump under a small pressure was now bubbled through the suspension for five minutes. This air was taken by the pump from a large room in the basement of the medical building; its oxygen content was slightly lower, and its carbon dioxide content slightly higher, than in outside air. The actual percentages were determined by later analysis. At the end of the equilibration period the tube, completely filled with the room air, and with the suspension, in gaseous equilibrium with this air, was closed off, leaving the contained gas completely saturated with water, at atmospheric pressure, and at approximately 25° C., the temperature of the room. The tube was then placed horizontally within a water bath at a temperature of 25° C. $\pm$ .2°. From time to time the tube was gently rocked by hand to keep the suspension approximately in gaseous equilibrium with the air above it. At the end of three hours the tube was removed and the suspension vigorously shaken into complete equilibrium with the gaseous phase. A sample of the contained gas was now withdrawn into a Bailey collector, and set aside for later analysis.

As quickly as possible the same suspension was again equilibrated with room air. The tube was then partially exhausted by a water pump, the residual pressure being measured by a mercury manometer connected with one inlet. Upon the attainment of the desired low pressure the stopcocks were closed, and the tube placed again within the water bath. At the conclusion of a second three hour period the gas in the tube was brought to atmospheric pressure and a sample collected. At the end of this second period the organisms were alive and active.

The gas samples were now analyzed by the use of a Haldane-Henderson gas analyser. Whenever possible duplicate or triplicate determinations were made, and the results averaged. Assuming the gaseous solubilities to be those given by the standard tables for pure water at this temperature, the total oxygen and carbon dioxide present at the beginning and at the end, in both air and water, were now calculated, the usual corrections for barometer, water vapor, etc., being applied.

The results obtained in fourteen experiments are given in Table I. It is seen that the oxygen intake is practically constant from 200 to 50 mm. Hg partial pressure of oxygen. Below 50 mm. the

values are somewhat reduced, but down to 11 mm. the intake is still at least 80 per cent. of that at atmospheric pressure. Since, in these experiments, an oxygen gradient must have been present from air to water, the actual tensions in the water were somewhat lower than those given in the table, which represent the tensions in the air. The ability of these organisms to utilize oxygen at low tensions therefore becomes even more evident.

TABLE I.

RESPIRATION OF *Paramecium* AT DIFFERENT OXYGEN TENSIONS.

Experiment.	Oxygen Pressure in Second Period.	Respiration in First Period.			Respiration in Second Period.			Ratio between O_2 Consumption in Second Period and that in First Period.
		O_2 Cons.	CO_2 Prod.	R. Q.	O_2 Cons.	CO_2 Prod.	R. Q.	
	mm. Hg.	*c.c.	c.c.		c.c.	c.c.		
1	208–192	1.030	.703	.683	1.027	.753	.733	.997
2	211–195	1.107	.663	.598	1.167	.762	.653	1.054
3	154–139	.969	.565	.583	1.025	.640	.625	1.058
4	154–135	1.345	.849	.632	1.390	.903	.649	1.033
5	122–109	.933	.763	.817	1.029	1.016	.986	1.103
6	92–68	2.088	1.490	.714	2.002	1.446	.722	.952
7	91–74	1.216	1.086	.893	1.245	1.204	.967	1.024
8	70–48	1.654	1.302	.787	1.612	1.448	.898	.975
9	70–60	.698	.390	.559	.724	.458	.633	1.037
10	70–57	1.131	.676	.598	.973	.553	.568	.860
11	60–42	1.645	1.028	.686	1.440	1.008	.699	.875
12	49–28	1.592	1.093	.686	1.546	1.115	.721	.971
13	28–11	1.146	.766	.668	.977	.638	.652	.853
14	28–11	1.642	1.134	.691	1.290	1.038	.804	.786
		Average R. Q.		.685			.736	

* Volume measured at 760 mm. Hg and 0° C.

The average of the respiratory quotients obtained in twenty-eight determinations comes out to be .710. Considering the wide range of the individual values it is hardly possible to attach any great significance to this figure, although it may be taken to suggest the presence of a fat metabolism under the conditions of the experiment, when the normal food supply is absent.

These preliminary experiments indicated that the method is applicable to such problems, but certain difficulties were encountered which made it advisable to complete the study on another material.

These consisted in (1) the impossibility of controlling the activity of the organisms, (2) the manipulation of gases at pressures very much below atmospheric, which prevented the exploration of very low oxygen tensions, and (3) the lack of complete gaseous equilibrium between air and water during the course of the experiment. The study was therefore continued with a modified method at Woods Hole on fertilized *Arbacia* eggs, which have no independent motility during the first hours of their development.

Experiments with Fertilized *Arbacia* Eggs.

In these experiments it has been found possible to secure two suspensions of eggs containing equal numbers of cells, whose respiratory exchanges check well with each other when the two are studied simultaneously under identical conditions. The eggs were freed from ovarian debris and body fluid and washed through several changes of sea water. A heavy suspension of cells was secured by permitting the eggs to sediment in a large beaker and then pouring off the greater part of the supernatant sea water. These were then fertilized. About ten minutes after fertilization two 60 cc. samples of this suspension were taken up by pipette and introduced into two tubes similar to that used for *Paramecium* but of a somewhat larger volume.

The lower oxygen tensions were secured by mixing oxygen and nitrogen, or air and nitrogen, in the desired proportions. Eight liters of such a gaseous mixture were collected in a large bottle, over water. One tube (*B*) was then brought into equilibrium with this mixture, the gas being bubbled through the suspension for at least five minutes. For the same period the second tube (*A*) was equilibrated with outside air. In every case a sample of gas was collected from the low pressure tube toward the end of the equilibration, and its later analysis accepted as giving the value of the initial oxygen and carbon dioxide percentages. The air which had passed through tube *A* was analyzed in several experiments and this value accepted for the rest as giving the initial oxygen and carbon dioxide percentages in the high pressure tube. It showed, after passing through the egg suspension, a slight diminution in oxygen and a slight increase in carbon dioxide.

At the conclusion of the equilibration the two tubes were closed

in such a manner that the contained gas was left at atmospheric pressure and at approximately 20° C. They were then placed side by side within a water bath, and rotated continually throughout the experiment, turning at the rate of about thirty times a minute. Under these conditions the eggs were always evenly distributed throughout the suspension, and kept in constant motion, the water was always nearly in equilibrium with the gas, and cleavage proceeded in a perfectly normal manner.

Running sea water was used in the water bath. Its temperature varied slightly from day to day. The lowest temperature recorded in any experiment was 18.2° C., the highest 20.2° C. The experiments continued in most cases for two hours; in a few cases for three hours. The first division occurs about one hour after fertilization at this temperature; subsequent divisions follow about every thirty minutes. At the end of the two-hour experiments the eggs were in the four and eight cell stage; at the end of the three-hour experiments they were in the sixteen and thirty-two cell stage. The material is not, therefore, unicellular throughout the whole experiment. The individual cells, however, in all of these early stages are all at the surface of the dividing egg in intimate relation with the oxygen supply in the water; there seems every reason to believe that the relationship under investigation will not be materially modified by this increase in number of cells, unaccompanied by any change in the mass of respiring tissue. We have reason to believe from the work of Gray (1925), that cleavage itself does not affect the rate of oxygen consumption, and that, after the first sharp rise following fertilization the consumption is practically constant during the first three hours of development. The unfertilized egg has so low a gaseous exchange that it has not proven practicable to follow its respiration by the present method.

At the end of the experiment samples of gas were secured from both tubes and analysed. The oxygen and carbon dioxide in the gas and in the sea water were then calculated for the beginning and for the end of the experiment. For this calculation the absorption coefficients for oxygen and carbon dioxide in sea water given in Tabulæ Biologicæ (Vol. 4, pp. 571–578) were used. The results of a typical experiment are as follows:

	Tube A.	Tube B.
Oxygen tensions during experiment.	155 to 142 mm. Hg.	61 to 49 mm. Hg.
Volume of tube	106.15 c.c.	105.39 c.c.
Volume of suspension	60 c.c.	60 c.c.
Gas Analysis at beginning:		
O_2	20.87%	8.22%
CO_2	.05%	.02%
N_2	79.08%	91.76%
Gas analysis at end (corrected for volume change):		
O_2	19.16%	6.59%
CO_2	.62%	.58%
N_2	79.08%	91.76%
Oxygen in air and water:		
At beginning	9.961 c.c.	3.863 c.c.
At end	9.147 c.c.	3.095 c.c.
Oxygen Consumption	.814 c.c.	.768 c.c.
Carbon dioxide in air and water:		
At beginning	.045 c.c.	.018 c.c.
At end	.558 c.c.	.517 c.c.
Carbon dioxide production	.513 c.c.	.499 c.c.
Volumes corrected to dry values at O° C. and 760 mm. Hg.		
Oxygen consumption	.741 c.c.	.699 c.c.
Carbon dioxide production	.467 c.c.	.454 c.c.
Respiratory quotient	.630	.649

Oxygen consumption at low pressure = 94.4% of that at atmospheric pressure.

Carbon dioxide production at low pressure = 97.3% of that at atmospheric pressure.

The results obtained in twenty experiments, carried out after the preliminary tests, are given in Table 2, and shown graphically in Fig. 1. The oxygen consumption is seen to be practically constant from an oxygen pressure of 228 mm. Hg. down to about 20 mm. Hg. Between 80 and 20 mm. there is a definite downward trend in the values, but at 20 mm. the consumption is still about 90 per cent. of that at atmospheric pressures. Below this point the consumption falls off sharply.

In Fig. 1 the experimental values are shown as rectangles. The height of this rectangle corresponds to 1 per cent. on the oxygen consumption scale; the length indicates the oxygen tension range in tube *B* during the course of the experiment. Each rectangle shows that over this range the oxygen consumption of the egg suspension in tube *B* was the indicated percentage of the consumption in tube *A*, run at atmospheric pressure. The absolute

TABLE II.

RESPIRATION OF FERTILIZED *Arbacia* EGGS AT DIFFERENT OXYGEN TENSIONS.

Experiment.	Oxygen Pressure in Tube B.	Respiration in Tube A.			Respiration in Tube B.			Ratio between O_2 Consumption in Tube B and that in Tube A.
		O_2 Cons.	CO_2 Prod.	R. Q.	O_2 Cons.	CO_2 Prod.	R. Q.	
	mm. Hg.	*c.c.	c.c.		c.c.	c.c.		
1	228.8–220.0	.423	.394	.931	.433	.294	.679	1.024
2	155.2–147.2	.473	.430	.909	.470	.386	.821	.994
3	152.2–144.6	.443	.329	.742	.447	.350	.783	1.009
4	142.0–135.7	.309	.257	.832	.315	.237	.753	1.019
5	123.2–112.4	.524	.436	.832	.533	.326	.611	1.017
6	116.8–104.3	.691	.443	.641	.733	.448	.611	1.061
7	85.5– 76.0	.650	.496	.763	.621	.403	.648	.955
8	70.6– 61.7	.572	.417	.729	.520	.412	.792	.909
9	66.6– 55.4	.665	.528	.794	.653	.493	.754	.982
10	61.2– 49.0	.741	.467	.630	.699	.454	.649	.944
11	44.6– 38.3	.390	.334	.856	.370	.339	.915	.949
12	36.8– 24.2	.818	.611	.768	.735	.535	.727	.899
13	30.0– 24.2	.406	.279	.688	.345	.217	.628	.850
14	23.9– 14.9	.592			.524			.885
15	23.7– 8.7	.856	.593	.697	.862	.558	.648	1.007
16	17.3– 10.1	.674	.444	.658	.419	.360	.859	.622
17	11.5– 6.3	.636	.543	.854	.367	.427	1.160	.577
18	7.9– 3.3	.565	.667	1.181	.268	.269	1.004	.457
19	7.1– .8	.746	.527	.706	.309	.488	1.582	.414
20	4.3– 1.7	.665	.445	.669	.151	.222	1.473	.227
		Average R. Q. .783			Average R. Q. (1–16) .725			

* Volume measured at 760 mm. Hg. and 0° C.

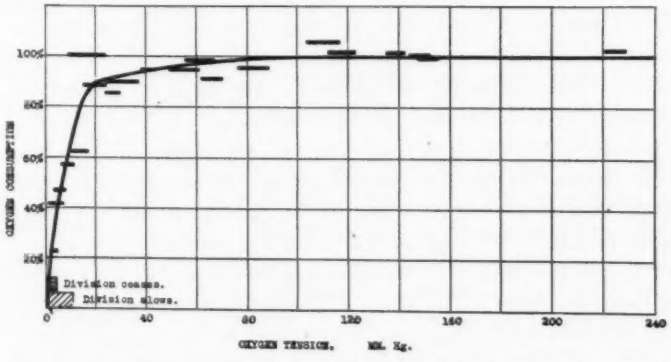

FIG. 1. Oxygen consumption of fertilized *Arbacia* eggs at different oxygen tensions. The range of tensions within which the division rate is affected is also graphically shown.

values vary considerably from experiment to experiment, but the graph of these percentages assumes a fairly regular and consistent form.

Correlated with the diminished oxygen intake at very low oxygen tensions retardation in development was observed in experiments 17–20. In all other experiments the eggs in the low pressure tube had developed as far as had those at atmospheric pressure. In every case 95–100 per cent. of the eggs developed. In experiment 17, continuing for two hours, a slight retardation in division rate was evident. Counts on 100 eggs from each suspension gave the following values:

	1-cell.	2-cell.	4-cell.	8-cell.
Tube *A* (High O_2)	4	6	57	33
Tube *B* (Low O_2)	5	14	74	7

In experiment 18 (2 hours) a more marked effect was observed. Counts on 100 eggs gave the following values:

	1-cell.	2-cell.	4-cell.	8-cell.	16-cell.
Tube *A* (High O_2)	2	0	52	40	6
Tube *B* (Low O_2)	54	39	7	0	0

In experiment 19 (3 hours) the eggs at atmospheric pressure were in the sixteen and thirty-two cell stage. In tube *B* about 80 per cent. had reached the four-cell stage, but none were found in later stages. In experiment 20 (2 hours) the eggs at atmospheric pressure were in the four and eight-cell stage. In tube *B* a careful search failed to reveal any cleavage whatsoever. It has long been known that in the complete absence of oxygen cleavage in these eggs is prevented. (See E. B. Harvey, 1926.) My own observations would suggest that a certain minimal concentration of oxygen is necessary for division, but the matter has not received a thorough study. The range of oxygen tensions within which development is either retarded or prevented is indicated graphically in Fig. 1. The values, taken from four experiments, are to be considered as approximations only. Taken in conjunction with the curve of oxygen consumption they show the great ability of

these eggs to carry out a normal development down to very low oxygen tensions.

It is of interest to note that in all four of these experiments in which retardation or inhibition of development occurred the respiratory quotient rose above unity; in experiments 19 and 20 the quotient reached the high values of 1.58 and 1.47. These figures suggest the presence of anaerobic respiratory processes at these low oxygen tensions. It is not possible to be certain concerning the matter, since, under these conditions of oxygen lack, acid metabolites may collect in the suspension and liberate carbon dioxide from the carbonates of the sea water.

In none of these experiments has the tension of carbon dioxide risen to such a point that it can have materially affected developmental rate. Haywood (1927) has shown that, in high concentration, carbon dioxide behaves as a narcotic and completely prevents cleavage when its tension rises above 230 mm. Hg. Below this value cleavage occurs at a rate slower than normal. The threshold tension for this carbon dioxide effect to appear was not determined, but it seems evident that at very much lower concentrations the retardation of development must become negligible. The highest carbon dioxide value observed in the present study was at the end of experiment 18, when the partial pressure reached 7 mm. Hg in tube *B*. The retardation of development observed at low oxygen tensions must therefore be caused by oxygen lack rather than by a narcotic effect of the carbon dioxide produced. Haywood also reports experiments on the influence of low oxygen tension upon developmental rate which agree with my own findings in showing practically no influence down to quite low values.

In most experiments carried out below an oxygen tension of 50 mm. Hg there was observed, at the end of the experiment, a liberation of pigment in the suspension in the low pressure tube which became more and more marked as the oxygen tension was lowered. This liberation of pigment apparently arose from the cytolysis of a certain number of cells. The actual percentage of eggs thus destroyed was not determined, but must have been small, since at the end of the experiment the volume of the eggs after sedimentation was not appreciably diminished. The downward

trend in the oxygen consumption values below 80 mm. Hg may be in part due to this destruction of a small number of the eggs, although we know, from the work of Warburg (1914) that respiratory exchanges may continue for some hours even in completely fragmented sea-urchin eggs, at a level not far below that found when the cells are intact.

The ability of both protozoa and sea-urchin eggs to carry on a normal respiratory exchange down to very low oxygen tensions points very definitely to the normal presence, within the cells, of a considerable oxygen tension. Oxygen is present in such amount that it does not limit the metabolism, whose rate is determined by other than oxidative reactions.

SUMMARY.

By standard methods of gas analysis the respiratory exchanges of *Paramecium* and of fertilized *Arbacia* eggs have been studied. The respiratory rate in both materials is found to be practically constant over a wide range of oxygen tensions, thus confirming older work done by other methods.

In the fertilized *Arbacia* egg the oxygen consumption is practically constant between 228 and 20 mm. Hg partial pressure of oxygen. Between 80 and 20 mm. Hg there appears to be a slight diminution in oxygen intake, but at 20 mm. Hg the consumption is still about 90 per cent. of that at atmospheric pressure. Below 20 mm. Hg the consumption is sharply reduced.

The cleavage of *Arbacia* eggs proceeds at a normal rate down to very low oxygen tensions. No retardation in development has been observed above 11 mm. Hg. Below this value the rate becomes slower and cleavage ceases entirely below 4 mm. Hg.

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A COMPARISON OF THE OXYGEN CONSUMPTION OF UNFERTILIZED AND FERTILIZED EGGS OF *FUNDULUS HETEROCLITUS*.

MARJORIE BOYD.

(From the Marine Biological Laboratory, Woods Hole.)

Since Warburg (1) in 1908 measured the oxygen consumption of *Arbacia* eggs and observed the marked increase following fertilization, the oxidation processes in marine eggs and embryos have been carefully investigated. The rate at which the oxygen is removed from the surrounding air or sea water has been correlated with the stages in development. Thus Shearer (2) measured the oxygen consumption during fertilization of Echinoderm eggs, and found a decided increase upon the addition of the sperm; "more oxygen is taken up in the first minute of the process than at any subsequent interval of the same time." In another article Shearer (3) states that, in the first hour of development, the fertilized egg consumes six to seven times as much oxygen as the unfertilized egg. In the star fish egg, however, according to Loeb and Wastenys (4) there is no increase in the oxidation rate after fertilization.

The rate of oxygen consumption is also correlated closely with heat production. Rogers and Cole (5) in their work on *Arbacia* eggs have shown how the heat production varies before, during, and after fertilization; according to them "the rate of heat production at the instant of fertilization is ten to twelve times that of the unfertilized egg."

The literature upon this subject reports work done almost exclusively upon invertebrate eggs. Apparently no previous study of the influence of fertilization upon respiratory rate has been made on any vertebrate egg. Scott and Kellicott (6) and Hyman (7), who have measured the oxygen consumption of *Fundulus* embryos at various stages of development, made no observations on the respiration during the first two hours after fertilization, and secured no information as to the influence of fertilization

itself. The present study represents an attempt to secure such information. It has been possible to show by several methods that fertilization markedly increases the oxygen consumption of the eggs of *Fundulus heteroclitus*. The time relations of this increase are of some interest.

1. Winkler Method for Determination of Dissolved Oxygen.

The Winkler method as applied to this problem was employed in the manner described by Amberson, Mayerson and Scott (8). 600 eggs were placed in 500 cc. of sea water in each of two small Erlenmeyer flasks. Samples for analysis were withdrawn through siphons. The water surface was covered with paraffin oil to minimize the diffusion of new oxygen from the air into the water. The sea water was analyzed for dissolved oxygen previous to experimentation; the initial sample was withdrawn after twenty to forty minutes. In order to secure successive determinations of the dissolved oxygen during an extended time, it was necessary to adopt a micro-Winkler method as suggested by Lund (9). For these analyses small vials of 6.5 cc. capacity were used. Fig. 1 represents the graph resulting from plotting the data shown in Table I. below. The values for the amount of oxygen consumed during a given period are obtained by subtracting the amount remaining in the sea water at the end of that period from the amount originally present in the sea water used for the experiment.

Table I.

Time.	Sea Water Originally Contains 5.1 cc. Oxygen per Liter.			
	Unfertilized Eggs.		Fertilized Eggs.	
	O_2 Remaining.	O_2 Consumed.	O_2 Remaining.	O_2 Consumed.
20 min.	5.0	0.1	4.7	0.4
45 min.	4.9	0.2	4.5	0.6
7 hrs.	—	—	3.7	1.4
10 hrs. and 35 min.	4.6	0.5	3.4	1.7
25 hrs. and 25 min.	3.8	1.3	2.8	2.3
31 hrs. and 25 min.	3.1	2.0	1.8	3.3

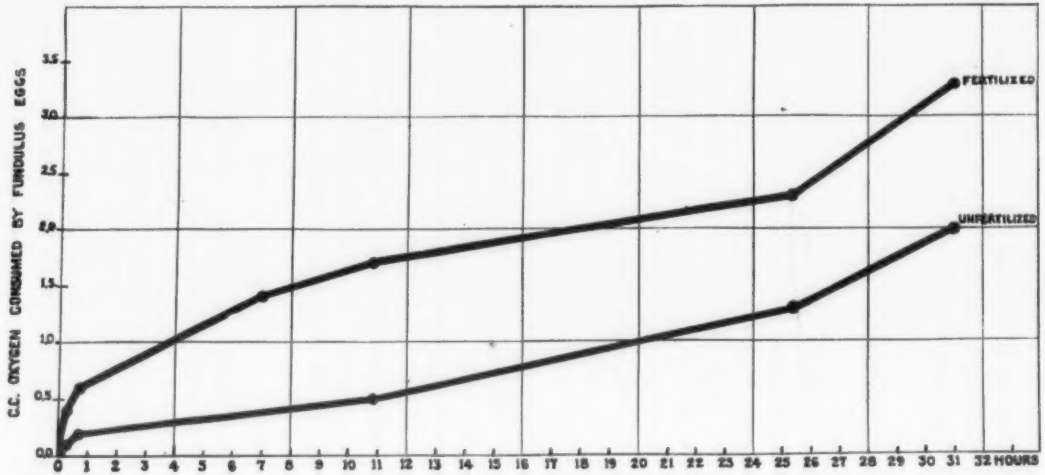

FIG. 1. Results obtained from Winkler Method. The amount of dissolved oxygen found at each successive time is subtracted from 5.1 c.c., the amount of dissolved oxygen originally in the sea water used for the experiment. The total amount of oxygen consumed is thus determined and is plotted against time.

It is to be noticed that the fertilized eggs had at the time of each determination consumed more oxygen than the unfertilized eggs. Data from similar experiments, as shown in Table II., show that the rate of oxygen consumption is most rapid during the first two hours following fertilization.

TABLE II.

Time.	Sea Water Originally Contains 5.2 cc. Oxygen per Liter.			
	Unfertilized Eggs.		Fertilized Eggs.	
	O_2 Remaining.	O_2 Consumed.	O_2 Remaining.	O_2 Consumed.
20 min. . . .	5.1	0.1	4.7	0.4
1 hr.	5.0	0.2	4.4	0.8
2 hrs.	4.9	0.3	4.0	1.2
4 hrs.	4.8	0.4	3.9	1.3

2. MICRO-RESPIROMETER METHOD FOR DETERMINATION OF OXYGEN.

The type of micro-respirometer that was used for the study of oxygen consumption by the *Fundulus* eggs is one that has been devised by W. O. Fenn for similar studies of *Arbacia* eggs. A small glass bottle with a ground glass neck is fitted with a ground glass stopper which is connected with a horizontal fine-bore manometer provided with a centimeter scale. In the center of the bottom of the bottle is a small compartment into which 0.5 cc. of 15 per cent. NaOH is introduced; the eggs to be studied are placed in the space surrounding the compartment. The NaOH serves to absorb the CO_2 produced by the eggs. A small drop of kerosene is introduced into the manometer and its movement across the tube from the outer to the inner end indicates both the amount of oxygen consumed and the rate of the process.

Five cc. of sea water, containing fifty *Fundulus* eggs, were pipetted into the micro-respirometer. Two micro-respirometers were used so that experiments on unfertilized and fertilized eggs could be carried out at the same time under identical conditions. The constants of each apparatus were found by calibration of the respective manometers. The micro-respirometers were immersed in a bath of running sea water; the temperature for all of the

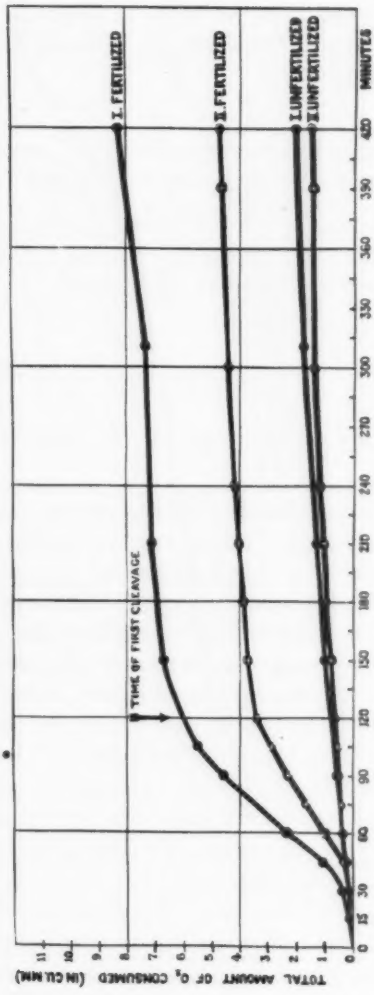

FIG. 2. Results obtained by the micro-respirometer method, showing total amounts of oxygen consumed by fertilized and unfertilized eggs at successive periods. Cubic mm. of oxygen are plotted against time. Experiments I. and II. are chosen to show typical results.

experiments proved to be $20.2 \pm .6°$ C. The readings of the meniscus of the kerosene drop were taken at intervals of fifteen minutes. Over twenty series were run, but in only the last five experiments were the temperature variations observed with a Beckman differential thermometer.

Figure 2 shows typical curves for the results obtained. In Experiment I. the number of cubic millimeters of oxygen consumed by the fertilized eggs is a little less than twice the corresponding amount in the case of the fertilized eggs in Experiment II. Nevertheless both curves show the same marked increase in oxygen consumption 45 minutes after fertilization. This increased oxygen utilization reaches a maximum during the period from 60 to 90 minutes following fertilization. From this time on, the amount of oxygen consumed per unit time falls so that the rate of utilization approximates that for the unfertilized eggs. It would appear, therefore, that the oxygen requirements of the unfertilized *Fundulus* eggs are increased by fertilization. The time relations of this increase are of theoretical interest; they are indicative of some oxidation process occurring within the egg for which an increased oxygen intake is a necessity. To follow the development in relation to the time, two control sets of 50 *Fundulus* eggs were placed in sea water and the stages of development were traced by microscopic inspection. It was found that the increased rate of oxidation occurs at a time before and during the appearance of the groove in the surface of the blastodisc which initiates the first cleavage. The subsequent cleavages evidently do not require such a marked rate of oxygen intake. A single run with twenty 9-day old *Fundulus* embryos revealed a later rise in the oxygen consumption which can be correlated with the marked rise in the rate which Scott and Kellicott found to occur at the time circulation is established.

To show still further the peculiarity of the time relations, the average amount of oxygen consumed per 10 minutes was calculated from the data of Experiment I. and is shown in Fig. 3. The difference in the rates of the fertilized and unfertilized eggs is markedly contrasted.

A few experiments were carried out by a third method and gave results that checked qualitatively with the two mentioned

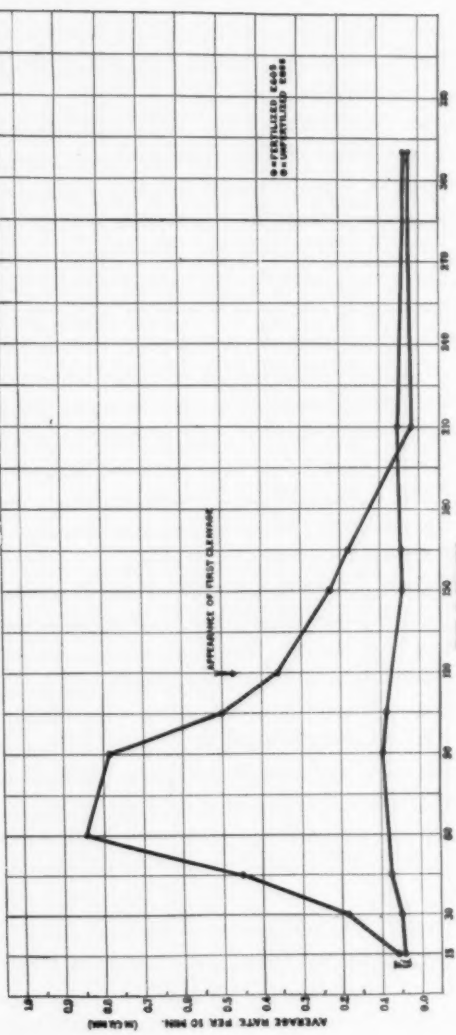

FIG. 3. Respiratory-rate-time graph. The marked increase in the rate of oxygen consumption after fertilization is shown to precede the time of first cleavage. The rate of oxygen consumption of unfertilized eggs is practically constant.

above. This method involved the analysis of air above 25 cc. of water in a 100 cc. Haldane gas collector. At the beginning of the experiment 200 *Fundulus* eggs were pipetted into each of two collectors with the sea water, and the water was equilibrated with atmospheric air which filled the vessel. The collectors were then sealed, immersed, and rotated in a bath of running sea water for two hours. More oxygen was found to have been lost from the sample of air taken from the collector containing the fertilized eggs than from that containing the unfertilized. This method proved to be only approximate as the rotation caused the eggs to stick together in a clump and normal development did not take place. The data secured gave a qualitative confirmation of the more accurate results obtained by the two other methods.

Summary.

By three methods it has been shown that the oxygen consumption of the eggs of *Fundulus heteroclitus* is greatly increased after fertilization. This increased rate of oxygen consumption is at its maximum from 60 to 90 minutes after the addition of the sperm, in a period immediately preceding the first cleavage. The oxygen consumption then falls to a level practically identical with that of the unfertilized eggs.

The writer wishes to express her appreciation to Dr. W. R. Amberson and Dr. W. O. Fenn for their suggestions, and to Mr. J. O. Pinkston for his assistance in the oxygen determinations.

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STUDIES ON *DALLASIA FRONTATA* STOKES.

I. POLYMORPHISM.

GARY N. CALKINS AND RACHEL BOWLING.

Dallasia frontata is a common fresh-water ciliate classified in the family Chiliferidæ, sub-order Trichostomina, order Holotrichida. It was originally described in 1886 by Stokes and characterized by him in 1888 as follows: "Body elongate-obvate, subcylindrical, transparent, longitudinally striate, and finely reticulated, five times as long as broad, the lower or ventral surface convex, the dorsal slightly concave, tapering posteriorly to a somewhat retractile tail-like prolongation forming about one-fifth of the entire body; anterior extremity narrowed, obtusely pointed; oral aperture narrow, ovate, obliquely placed on the ventral or convex surface at some distance from the anterior extremity, enclosing two small vibratile membranes; contractile vacuole single, spherical, near the center of the dorsal or concave border; nucleus presumably represented by large, ovate, sub-central clear space. Length of body 1/180 of an inch. Habitat.—Still water, with *Myriophyllum*" (1888, p. 171).

This characterization is not adequate to describe the many form changes which this remarkable organism passes through in its life history, changes which we are not yet ready to interpret as to cause or full significance, but which we will describe in the following pages.

The organism may be found without much difficulty in the water of Van Cortlandt pond in the environs of New York. Unlike *Uroleptus mobilis* it cannot be regarded as a rare form and is probably widely distributed in fresh-water ponds throughout the country. Many individuals were encountered in the autumn of 1927 and individuals were isolated in different types of media in isolation culture dishes usually employed for this work. Initial experiments with culture media including pond water, hay infusion, and the combination of hay and flour soon showed that the latter, as

in the case of *Uroleptus mobilis,* was the most favorable. This medium, made up in the same way as for *Uroleptus mobilis* during eight years of culture, has been consistently used for some of our material since October 6, 1927. Later, media made up with rice and with cracked wheat were tried and some of our material is now successfully running on the latter. In this, as in the hay-flour medium, individuals in the period of maximum vitality divide from four to six times in twenty-four hours.

The material of the isolation cultures is run in "series" and "lines." A series is made up of all the progeny of a single individual isolated as an ex-conjugant; lines, usually five in number, are isolation cultures made from the first five individuals formed by division of the ex-conjugant. Individuals from each line are picked up with a capillary pipette and transferred daily to another culture dish of fresh medium. After such isolations are made the unused individuals of a series are transferred to a Syracuse dish containing about 10 cc. of fresh medium. Such reserve material is allowed to multiply with no change of the medium for from six to ten days. It constitutes a "conjugation test" such as proved successful with *Uroleptus mobilis.* In this way abundance of material is available for study. With Dallasia after a few weeks, epidemics of conjugation occurred in the Syracuse dishes and pedigreed series were started.

Material for preparations has been fixed in osmic fumes, Flemming's, Hermann's and Schaudinn's fluids. The latter, made up as a saturated solution in 95 per cent. alcohol is most satisfactory for general staining. Iron haematoxylin is good for general topography but inner cellular structures are obscured by the dense cortical zone of deeply staining granules. This however, may be avoided by prolonged treatment with turpentine. Auerbach's combination of methyl green and acid fuchsine (without orange G) is excellent for cortical structures and for the mouth parts. Vital stains are useful for demonstrating some structures particularly the capsules about the "couples."

The derived organization of *Dallasia* is so delicately adjusted to its environment that small changes in the latter cause remarkable changes in form. This leads to polymorphism which, more than with any other free-living protozoön known to us, is character-

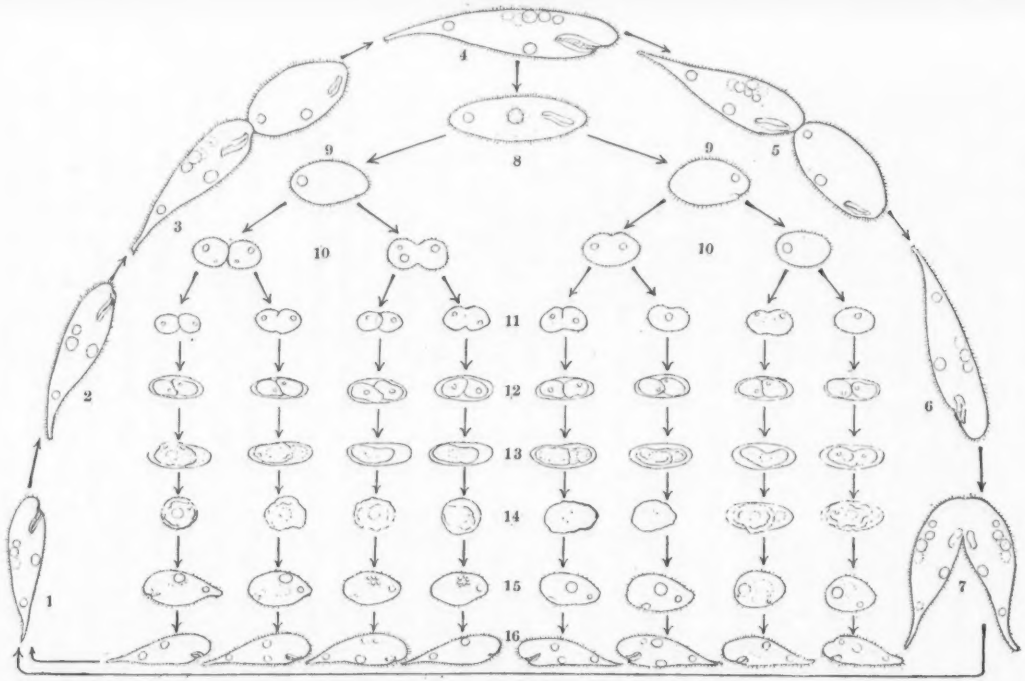

FIG. I. *Dallasia frontata* Stokes. Camera lucida sketches from living organisms. Same magnification throughout. Life cycle. 1, 2. Young tailed forms. 3, 5. Division of tailed form. 6. Tailed form in agglomeration stage, ready for conjugation. 7. Conjugation of tailed forms. 8. Boat type derived from tailed form. 9. First division of boat. 10. Second division of boat without growth, and third division under way. 11, 12. Fourth division of boat forming sixteen individuals which become encapsulated in pairs of sister cells. 13, 14. Fusion of sister cells in capsules, forming eight zygotes. 15, 16. Development of zygotes into small tailed forms.

istic of this ciliate. Certain well-marked types of organization follow in the same order. To these we have applied purely colloquial names which have no resounding classical roots indeed, but which enable us to distinguish clearly between the forms indicated by them. These forms are (1) tails; (2) boats; (3) couples (gametes); and (4) pairs, and they will be described in this order.

1. *Tails.*—This term is an abbreviation for "tail-bearing forms" such as indicated by the original description of Stokes. They are relatively large (105μ to 140μ × 22μ to 36μ) and, owing to the remarkable mouth have a curious resemblance to a microscopic shark a resemblance to which Stokes called attention. The anterior end is rounded and in most cases this is the broadest part of the organism which tapers gradually to the posterior end where it narrows into a well-marked tail (Fig. I., 4 and Fig. III., 1). The tail is quite variable in length and shape. Sometimes it is long, resembling the handle of a skillet; again it is reduced until it is little more than the sharply-pointed posterior end of the cell. In other cases the tail disappears entirely. These forms are fairly sluggish, richly stored with food, and are usually attached to the substratum by the tip of the tail where they swing about in circles with the tips of the tails as centers. The cilia are long and closely set in longitudinal rows of which there are about forty.

Another type of tailed form is much longer and somewhat more slender but unlike the fat form is very active and rarely becomes attached.

So far as the visible structures are concerned the most complex part of the organism is the mouth. (Fig. II., 1). This is relatively large and lies in the anterior quarter of the cell. The external aperture varies in shape from an elongated slit to a circular opening. It leads into a spacious buccal pouch (*B. p.*) which extends inwards and diagonally from a region slightly anterior to the mouth, to the gullet which is posterior to the mouth. The entire apparatus is about 27μ long and 15μ wide, thus taking up about one-fifth of the organism. On the floor of the buccal pouch is a long tongue (*T*), triangular in cross section, which runs almost the full length of the pouch (Fig. II.). On one side of this and at the anterior end is a broad endoral membrane which fre-

quently protrudes from the mouth (Fig. II., *E.m.*). At the region of the gullet and on the opposite side of the tongue is a long, narrow undulating membrane, the adoral membrane (Fig. II. *A.m.*). From the base of this membrane to the gullet is a long ladder-like structure recalling the "railroad track" of Chlamydodon (*A.c*). There is evidence of a complicated neuro-motor system which will be described in a later paper on the cytology of these forms.

The macronucleus, like the organism, is polymorphic. In many individuals it appears to be emarginate, frayed out and of a de-

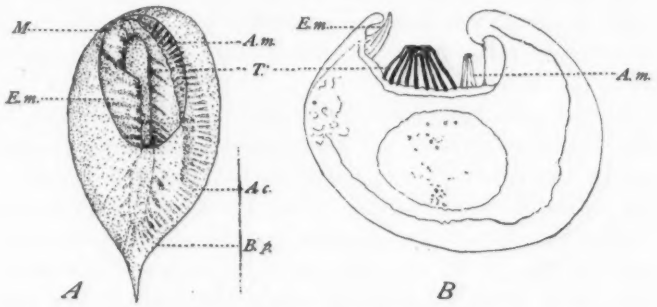

FIG. II. Mouth and buccal pouch of *Dallasia frontata*.
A. Total preparation of tailed form; mouth and buccal pouch only.
B. Transverse section of tailed form.
A.c., ladder; *A.m.*, adoral membrane; *B.p.*, buccal pouch; *E.m.*, endoral membrane; *M.*, mouth opening; *T.*, tongue and supporting bars.

cidedly unhealthy appearance. It is often splinter-like and irregular, but at the approach of division it becomes more condensed and homogeneous and ellipsoidal in form. It divides without mitosis and in the characteristic manner of most macronuclei.

The micronucleus is usually single, spherical, and homogeneous, and is closely applied to the macronucleus. It divides by mitosis.

The contractile vacuole is a single vesicle, in the middle of the ventral surface; feeding canals are absent but a ring of feeding vesicles, clearly visible after contraction of the vacuole, are present.

The cytoplasm is filled with great vacuoles which are frequently so abundant as to distort the organism. They are gastric vacuoles for the most part but are frequently merely fluid-filled vesicles.

Granules of large size and great number are present in all stages of these tailed forms and are a nuisance in preparations stained with iron haematoxylin. The majority of them stain well with the vital dyes neutral red, brilliant cresyl blue and methylene blue.

All in all these tailed forms are remarkably variable in size and shape. They appear to be highly sensitive to environmental stimuli readily becoming amorphous and variously distorted. If the medium is too rich such distortions are more numerous. For some unaccountable reason, possibly because of incomplete reorganization after division, minute dwarf forms with grotesquely large mouths are frequently seen (Fig. III., 4). Such types are prone to change into distinctly amoeboid forms with protoplasmic processes which cannot be distinguished from pseudopodia (Fig. III., 4*a*).

2. *Boats.*—In form and character boats are quite different from the tailed forms. They are considerably smaller (68μ to 83μ) and are derived from the tailed forms by gradual absorption of the attenuated caudal extremity. Both anterior and posterior ends become rounded and the organism becomes navicular and symmetrical (Fig. I., 8; Fig. III., 10). The environmental conditions under which the transition occurs have not yet been fully determined but the period required for it varies according to the age of the series. It is a striking phenomenon to see a rich stock culture in fresh medium yield nothing but boats twenty-four hours later. Such boats are not attached but shoot about the culture dish with amazing speed. After another 24 hours the majority of them have divided four times, giving rise to sixteen minute cells which separate off in pairs to form the couples. After the first division of the boats the daughter cells (gamonts number 1) are more quiet than the original boat and have a tendency to rest on the bottom where the second division takes place. The daughter cells of this second division (gamonts number 2) still have the ability to move but their movement is more or less spasmodic and irregular and their daughter cells (gametocytes) derived from a third division, are now quiescent (Figs. I., 10, and III., 13). These forms, however, are rarely found on the bottom but, together with the couples, are suspended in the medium.

The early stages of the boats show mouth parts only slightly

FIG. III. *Dallasia frontata* Stokes. Life cycle. Camera lucida sketches from preparations. Same magnification throughout.

1. Vegetative individual from isolation culture.
2. Boat-shaped individual before tail is formed which may originate at any time from the anterior end of dividing tailed form.
3. Early stage of division of tailed form.
4. Degeneration type of tailed form which may give rise to an amœboid form 4*a*.
5, 6. Later stages of division of tailed form.
7. Conjugation.
8, 9. Ex-conjugants which reorganize into tailed forms.
10. Typical boat-shaped form characteristic of mass cultures after two to three days.
11. First binary division of boat.
12. Second division of boat.
13. Third division of boat giving eight products.
14, 15. Fourth division of boat and fusion of pædogamous gametes in capsules to form eight zygotes.
16. Development of zygote into young individual.
17. Intermediate young individuals from couples.

different from those of the tailed forms but these parts are slowly absorbed and in the second generation of the boats they have entirely disappeared. The macronucleus of the boat form is more condensed, more definite in form, and stains more readily than in the tailed form, and this intensity of staining is retained throughout all of the later stages. The protoplasm likewise is denser and has lost its included vacuoles but there are still many granules which are partitioned out at each division with apparently no increase in their total number. The contractile vacuole varies considerably in position, sometimes on the ventral surface, sometimes on the dorsal and frequently nearer one pole than the other. In the third and fourth divisions of the boats the onset of division is always indicated by the presence of two vacuoles symmetrically placed in the cell.

The first two divisions are fairly slow, requiring several hours but the last two divisions follow one another in quick succession.

Boat-shaped forms may appear at any stage and appear to be a palingenetic phase of the organism. Thus in ordinary division of the tailed form the anterior half is navicular until the tail is regenerated. Also just as pathological tailed forms turn into amœboid cells so the boat-like individuals may undergo a similar pathological change. In some cultures the entire population apparently becomes thus transformed into amœbæ.

3. *Couples.*—The first two divisions of a "boat" lead to small individuals (21μ to 25μ) of broadly ellipsoidal form and with relatively large nuclei (Fig. III., 12). The daughter cells formed by the second division still have the power to move and usually become widely separated. Each divides into two and these two quickly give rise to four. Chains of four cells are characteristic and as there is a tendency at this period for the boats and their products to agglomerate, great masses of these chains are frequently found in the Syracuse dishes. The four cells of a chain soon become associated as two pairs and these are the "couples" (functionally gametes) of our terminology. These pairs measure from 22μ to 26μ, each individual, from 11μ to 13μ. About each couple is a delicate capsular membrane resembling a fertilization membrane, but there is as yet no fertilization, hence the resemblance is closer to a sporocyst membrane of two gregarines in pseudo-conjugation (Fig. I., 13; Fig. III., 14).

We have repeatedly watched the process of couple formation in the living cells and the further changes which take place within the capsule. An instructive picture is obtained by use of neutral red which stains some of the endoplasmic granules and these furnish points of orientation. The two cells of a couple fuse to form a zygote (Figs. I., 13, and III., 15). The nuclei also fuse. We have watched this fusion in living couples under an immersion lens and have noted a center in each gamete where brownian movement of granules is evident. Stained preparations show that these centers are nuclei. After fusion of the cell bodies these centers approach and melt into one immediately after which there is a more violent brownian movement of the granules.

These activities show that the boats are gamonts which give rise to gametocytes and the latter to gametes of which there are sixteen from each gamont. Fertilization is strictly paedogamous and nothing like it has been described for any type of ciliate. The nearest approach to it is Brumpt's account of encystment and fusion in *Balantidium coli,* but here two gamonts come together, no gametes are formed and the two individuals, as hologametes, fuse within a membrane analogous to the sporocyst membrane of gregarines.

This period of copulation is a critical one in the history of *Dallasia.* Up to the present time we have not succeeded in rearing a single zygote in isolation culture. Many young forms are found in the Syracuse dishes in which an epidemic of copulations has occurred (Fig. I., 16); some of these are not yet provided with mouths and their development into mouth-bearing forms has been repeatedly observed (Fig. I., 15). The origin of these young forms from the stage of the encapsulated zygote has also been observed but we have not yet succeeded in providing a suitable environment for their continued life in isolation culture. In many cases, but not in all, the zygote apparently encysts within the capsule (Fig. III., 15*d*) and such cysts are liberated by the dissolution of the capsular membrane. The further fate of these cysts is unknown.

4. *Pairs or Conjugants.*—We have cultivated *Dallasia* in isolation cultures for four months and now have eight series of different ages under observation each series derived from an indi-

vidual ex-conjugant. Pædogamous copulation, described above, occurs in conjugation tests made within a week of the first division of an ex-conjugant and epidemics of such unions still occur at intervals in our oldest series. They occur less frequently and in much milder form when the individuals of a series are mature for conjugation.

Conjugation epidemics are rare. Tests have been made daily by placing the reserve individuals left over after the usual isolations are made, in a Syracuse dish with about 10 cc. of fresh medium. These dishes are set aside in a moist chamber and left for at least one week and usually without the addition of fresh medium. They are examined daily and the observations recorded. In the early life of a series boats usually appear within two or three days and the boats usually give rise to couples. If, however, such boats are transferred to fresh medium they change again into tails. Sometimes fully 100 per cent. of the original tailed forms change into boats and these into couples and zygotes but as a series grows older there is an increasing percentage of tailed forms which do not become transformed into boats and an increasingly diminishing number of couples. In Syracuse dishes with material from older series there is thus a predominance of tailed forms at all stages. These are somewhat smaller (Fig. I., 6) than are the individuals of the isolation cultures and they show the same type of agglomeration as does *Uroleptus mobilis* in similar conjugation tests. As with *Uroleptus* such agglomerations are usually although not invariably, followed by conjugation of the individuals.

The first epidemic of conjugations occurred after thirty-five days of culture of a wild individual and gave us material for Series 2 and 2*a* of our pedigreed races. In one of these (2*a*) a mild epidemic occurred in the 96th generation or 25 days after the first division of the original ex-conjugant, and Series 3 and 4 were derived from it. A second epidemic occurred in the 160th generation or 47 days after the first division of the ex-conjugant and from this epidemic Series 5 and 6 were started. Three other epidemics have appeared in Series 3 and 4 and have furnished material for Series 7 and 8.

The conjugating individuals are relatively small (77μ to 102μ) and are always tailed forms. Union occurs as in *Uroleptus* or

Spathidium, etc., at the anterior ends and, again as in *Uroleptus* the mouth parts are not involved. The mouths, however, are greatly reduced and apparently are absorbed, new ones being formed by the ex-conjugants. The period of actual fusion varies from twelve to twenty-four hours and the period of reorganization of the ex-conjugant varies from one to four days. The cytological details have not yet been worked out but meiotic divisions, interchange and fusion of nuclei appear to follow the customary history.

The average division rate for the initial 10-day period is high and is higher in most cases than the division rate for the same calendar period of the parent series. As with *Uroleptus*, however, this is not invariable as the following table shows:

Series 2*a*	division rate		1st.	10	days,	38.6.	Parent series	same	period		19.2
Series 3	"	"	"	"	"	36.8.	"	"	"	"	40.2
Series 4	"	"	"	"	"	40.8.	"	"	"	"	40.2
Series 5	"	"	"	"	"	29.6.	"	"	"	"	24.4
Series 6	"	"	"	"	"	33.4.	"	"	"	"	25.2

It is too early to draw any conclusions as regards vitality before and after conjugation, this subject will be discussed in a later study.

Discussion.

So far as we are aware *Dallasia frontata* presents a unique phenomenon hitherto undescribed for the ciliated protozoa. This is the interpolation of a paedogamous fertilization stage in the otherwise ordinary cycle from ex-conjugant to conjugant. Two distinct and entirely different fertilization phenomena in the same life cycle certainly furnish food for reflection, particularly as regards the significance of fertilization in general. The nearest parallel case that we know is *Cryptochilum echini*, as described by Russo. The high death rate, in cultures, after copulation may be significant. It may mean that the culture medium is not suitable for this stage of the organism or it may mean that the encapsulated stage is taken into some other organism where part of the life history is spent as a parasite or as a commensal. Further study of the organisms in culture with experiments to test the effect of different media, which are now under way, may throw more light on this problem.

The novelty of *Dallasia* does not lie in the copulation of micro-

gametes; this phenomenon is known in the Opalinidae. Nor does it lie in the union of paedogamous gametes as this phenomenon is well established in the case of *Actinophrys sol* and in the case of *Actinosphaerium eichhornii*. There is certainly no novelty in the phenomenon of conjugation of *Dallasia* for in this it agrees with the vast majority of ciliates. The novelty lies in the combination of fertilization by copulation and fertilization by conjugation.

It is well known through isolation culture work with infusoria that a reorganization process without union of individuals occurs and has the same effect on vitality as does conjugation. It is a process of parthenogenesis termed endomixis by Woodruff and Erdmann (1914); and in some form or other it occurs in practically every ciliate that has been studied. It takes place prior to and during the early phases of encystment in the Hypotrichida, without encystment in various species of *Paramecium*. In *Uroleptus mobilis* endomixis with encystment is a characteristic phenomenon of the early stages of the life cycle (Calkins, 1926); it becomes infrequent with maturity of the protoplasm and is absent altogether in the later stages. In *Dallasia frontata* the incidence of couple formation in the early stages of the life cycle, the formation of capsules, together with the absence of any evidence up to the present, of the ordinary forms of endomixis, lead us to the conclusion that we have here a very unusual, perhaps primitive, type of endomixis. If this conclusion is correct the further hypothesis is permissible that endomixis as ordinarily observed in ciliates is a reminiscence of ancestral gamete-brood formation.

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THE BACTERIOLOGICAL STERILIZATION OF *PARAMECIUM*.

ARTHUR K. PARPART.

(From the Biological Laboratory, Amherst College, Amherst, Mass.)

I.

If for one reason or another it is necessary to control the bacterial content of the medium in which *Paramecium* is living, the first step is a reliable method for the bacteriological sterilization of the animals.

Hargitt and Fray ('17) devised a method which they believed accomplished this end. Their procedure, in brief, consisted in transferring a single animal, by means of sterile pipettes, through five successive washings of sterile water contained in sterile depression slides, the latter being enclosed in Petri plates. There is no evidence in their paper as to how many animals were treated in this way to determine the efficiency of the method. According to Philipps ('22) the technique of Hargitt and Fray "is undoubtedly reliable." However, she used a procedure in her experiments which "made it necessary to wash each animal seven times instead of five."

II.

In his first attempts to sterilize *Paramecia* the present writer increased the number of washings to ten. The animals were obtained from a pedigreed culture of *Paramecium caudatum*, growing on a 0.7 per cent. infusion of pure timothy hay in tap water. This same solution was used for washing. The solution was sterilized in an autoclave at 12 to 15 pounds pressure for 45 minutes.[1]

As a first step eight animals were washed ten times, with the purpose of determining, first, the diminution in the number of

[1] The difficulty, experienced by Hargitt and Fray, of getting *Paramecia* to live on media sterilized in an autoclave under high pressure, has never been encountered in these experiments though pure lines of *Paramecia* have been carried for a number of months on hay infusions and beef extracts treated in this way.

bacteria that occurred during the 1st, 3d, 5th, and 7th washes; second, the number of animals sterile in the 10th wash. To accomplish the first purpose, the 1st, 3d, 5th, and 7th wash fluids were plated, these plates incubated at 37.5 degrees C. for 72 hours and examined. For the second purpose the 10th wash fluid together with the animal was broth cultured, and the cultures treated in the same way as the plates. The results are recorded in Table I.

TABLE I.

REDUCTION IN NUMBER OF BACTERIA IN WASHES, 1, 3, 5, AND 7. EFFICACY OF 10 SUCCESSIVE WASHINGS.

Animal No.	Number of Colonies on Plates of wash Fluid No.				Broth Culture of Wash No. 10 + Animal.
	1	3	5	7	
1	9,000	1	0	0	Infected
2	6,000	3	0	0	Infected
3	10,000	0	0	0	Sterile
4	9,000	2	0	0	Infected
5	13,000	0	0	0	Sterile
6	9,000	0	0	0	Infected
7	16,000	2	0	0	Infected
8	16,000	0	0	0	Infected

The diminution of the number of bacteria in successive washes up through the 5th, as brought out in the above table, concurs very well with the results obtained by Hargitt and Fray. The discrepancy between the number of bacteria present in their first wash and the infection I found may be accounted for by the fact that they transferred only a small portion of these washes to agar plates, while I transferred the entire amount.

Superficially the fact that no animals contaminated the 5th wash might be taken to indicate their sterility. However, 80 per cent. do contaminate the 10th wash. These, of course, could not have been sterile at the time of the 5th washing.

To further test this particular point 18 animals were washed 10 times, and the 10th wash fluid together with the animal broth cultured. The latter was incubated at 37.5 degrees C. for 72 hours and examined. The results are tabulated in Table II.

Of the 26 animals included in these tables only 5 were sterile in the 10th wash.

TABLE II.

EFFICACY OF 10 SUCCESSIVE WASHINGS.

Total Number of Animals Tested.	Broth Cultures of the 10th Wash Fluids + the animals.	
	Sterile.	Infected.
18	3	16

III.

The Hargitt and Fray sterilization method differs from the above method in that they employed a sterile tap water solution for the washing and passed the animals through only 5 wash fluids. Conceivably sterile tap water might be a better sterilizing agent. At any rate, it speeds up animals put into it and leads to rapid reversals which possibly enable the *Paramecia* to throw off more readily the bacteria lodged between their cilia.

Accordingly, 30 animals were washed 5 times in sterile tap water. Broth cultures of the 3d wash fluid and the 5th together with the animal were incubated at 37.5 degrees C. for 72 hours. The results are tabulated in Table III.

TABLE III.

EFFICACY OF 5 SUCCESSIVE WASHINGS.

Total Number of Animals Tested.	Broth Cultures of the 3d Wash Fluids.		Broth Cultures of the 5th Wash Fluids + the Animals.	
	Sterile.	Infected.	Sterile.	Infected.
30	28	2	3	27

Only one conclusion is possible. In the majority of cases 5 and even 10 washings in sterile media cannot be relied upon to sterilize a *Paramecium*.

IV.

As washing is the only practical method for ridding *Paramecia* of bacteria, the following technique was devised.

1. The washings were performed in depression slides, each slide being enclosed in a Petri plate. Those plates in which *Paramecia* were cultured had a thin glass slide under the de-

pression slide, so that the sterile water poured into the plates to make them serve as moist chambers could not get into the depression.

2. The pipettes for transferring the animals through successive washes were made from soft glass tubing having an inner diameter of 2 mm. and a wall of 1 mm. thickness, by drawing this out to capillary fineness. Of the 60 pipettes made in this manner, 10 were chosen at random and the inner diameter measured at the tip of the capillary portion. The average inner diameter was 213 micra; none varied more than 30 micra from this average. The large end was plugged with cotton, and each pipette plugged into a separate test tube.

3. The sterilization of the pipettes and of the depression slides in Petri plates was carried out in a dry oven at between 160 and 170 degrees C. for 45 minutes.

4. The actual washing of the *Paramecia* was performed under a hood which was placed at one end of a large table, and consisted of a wooden frame (3 ft. by 15 in. by 11 in.), with a glass top and cloth sides. The front cloth, which served as entrance, was loose at the bottom. Toward one end there was a binocular microscope with sufficient focal length so that its oculars extended through and above the top. Cloth, with slits in it for the oculars, was glued to the edges of the glass surrounding the oculars.

In handling the animals the transfer pipettes were attached to a rubber tube plugged with cotton and operated by means of mouth suction while the operator was looking through the oculars.

5. All of the various types of culture and wash media used were put into separate, one-liter flasks fitted with glass siphon tubes. Rubber tubing with a glass pipette at one end led off from each siphon tube. After the flasks, tubing and pipettes (the latter plugged into small test tubes) had been sterilized in an autoclave at 12 to 15 pounds pressure for 45 minutes and the corks surrounding the siphon tubes sealed with paraffin, they were arranged outside of the hood and the rubber tubing and pipette led through the back into the hood. The pipettes were suspended at the back of the hood in such a way that their tips, after the test tubes had been removed, did not touch anything. By use of carefully adjusted pinch clamps the size of the drops flowing from

these pipettes was regulated, and hence the volume of media could be determined. The fact that none of the culture media thus treated became infected, although the pipettes were exposed continually for a number of months to the air of the hood, is very good evidence of the efficiency of the hood.

6. The wash fluid was prepared by placing 250 mg. of Liebig's beef into 200 cc. of sterile tap water. This solution was bacterized from the cultures of the pedigreed series of *P. caudatum* being cultured on 0.25 per cent. beef extract, incubated at 37.5 degrees for 2 days and diluted up to 1 liter with tap water. It was placed in one of the liter flasks, sterilized and arranged for use.

7. The actual steps in the washing of an animal were:

(*a*) Three piles of 5 Petri plates each were placed under the hood, and 6 drops (about ⅓ cc.) of wash fluid was put into each of the enclosed depression slides. The lowermost Petri plates served as moist chambers for the 5th wash and hence contained slides under the depression slides.

(*b*) The 15 pipettes necessary for the transfers were placed under the hood.

(*c*) The culture containing the *Paramecia* was placed on the microscope stand and a single individual transferred to the uppermost slide in each stack of plates. Each animal was transferred successively to the depression slide in the Petri plate immediately beneath. By working in rotation from stack to stack, the animals remained in each wash about one minute.

(*d*) When all three animals were in the 5th wash, from 3 to 4 cc. of sterile distilled water was added to the lowermost plates. This prevented excess evaporation from the depression slide while the 5th wash fluid and the animals were being incubated for 5 hours at 25 degrees C.

(*e*) At the end of 5 hours, each animal was again transferred through 4 washes.

(*f*) From the last of these, the 9th, the animal was transferred to the desired culture media. The Petri plate of this, the 10th wash, was converted into a moist chamber as above (*d*).

V.

The data demonstrating the efficiency of this method are summarized in Table IV. In this summary are included the data on those animals which were placed on some type of sterile medium after the 9th wash. The data were obtained by broth culturing the 5th wash after the animal had been in it 5 hours, and the 10th wash together with the animal after the latter had died in it. The death, in some cases, came only after a number of days, during which time the 10th wash and the animal were incubated at 25 degrees C. and examined every 24 hours until the death of the animal. The broth cultures were incubated at 37.5 degrees C. for 72 hours before being examined.

TABLE IV.

SUCCESS OF 10 WASHINGS; THE ANIMAL REMAINING IN THE 5th WASH FIVE HOURS.

Total Number of Animals Tested.	Broth Cultures of the 5th Wash Fluids.		Broth Cultures of the 10th Wash Fluids + the Animals.	
	Sterile.	Infected.	Sterile.	Infected.
50	17	33	50	0

The number of animals tested and the fact that all were sterile in the 10th wash shows conclusively that the method adopted will rid *Paramecia* of bacteria.

The length of time that an animal is allowed to remain in the 5th wash fluid is a significant factor in accomplishing the sterilization. It raises the question as to why many animals shed bacteria into the 5th wash fluid, but a few do not.

VI.

Tables III. and IV. appear to demonstrate that *Paramecium caudatum* defecates bacterial spores. According to Table III., over 93 per cent. of the *Paramecia* were no longer shedding bacteria into the 3d wash fluid, yet over 90 per cent. of these later proved infected. Table IV. shows that 66 per cent. of the animals left in the 5th wash for 5 hours shed bacteria, yet when these animals were washed four times more they proved sterile.

If the majority of *Paramecia* can be passed through a 3d wash fluid without shedding any more bacteria, and then later do so, it seems highly improbable that the bacteria are on the outside of the animals.

To test this point the following experiments were performed. Seventeen bacteriologically sterile *Paramecia* were left for 24 hours in a pure culture of *Bacillus prodigiosus* in beef extract, while 21 sterile animals were left for the same length of time in a pure culture of *Bacillus subtilis* in beef extract. At the end of this time the animals were washed, individually, ten times; the time of the 5th wash fluid being varied. Those fed on *B. prodigiosus* were allowed to remain in the 5th wash for ½ hour. Those fed on *B. subtilis* were left in the 5th wash from 2 to 5 hours. About 24 hours after the animals had been put into the 10th wash, this together with the animal was transferred to a broth culture, incubated at 37.5 degrees C. for 72 hours and examined. The results are tabulated in Table V.

TABLE V.

EXCRETION OF SPORES BY *Paramecia* FED ON *B. subtilis*.

Total Number of Animals Tested.	Bacterial Culture on Which Animal Was Placed.	Time Each Animal Spent in the 5th Wash.	Broth Cultures of the 10th Wash Fluids + the Animals.	
			Sterile.	Infected.
17	*B. prodigiosus*	½ hour	17	0
4	*B. subtilis*	1 hour	1	3
7	*B. subtilis*	2 hours	1	6
4	*B. subtilis*	3 hours	1	3
6	*B. subtilis*	5 hours	6	0

Bacillus prodigiosus has never been known to produce endospores, while *Bacillus subtilis* produces endospores very readily.

In every case the *Paramecia* that had been cultured in *B. prodigiosus* were able to throw off all bacteria, although the animals remained in the 5th wash fluid only ½ hour. Eighty per cent. of the *Paramecia* cultured on *B. subtilis* and then left in the 5th wash fluid up to 3 hours were not sterile when washed five times more.

Since both of these species of bacteria have the same type of

flagellae, it seems very unlikely that *B. subtilis* could remain lodged between the cilia of the *Paramecia* for a longer period of time than *B. prodigiosus*.

The above data seem to clearly indicate that *Paramecium* defecates solid material, in this case bacterial spores.

The efficiency of the sterilization technique adopted is further attested by the data in Table V. Those animals washed after having been cultured in *B. subtilis*, and left in the 5th wash for 5 hours, were all sterile in the 10th wash.

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THE EFFECT OF MATERNAL AGE AND OF TEMPERATURE CHANGE IN SECONDARY NON-DISJUNCTION.

R. R. HUESTIS,

UNIVERSITY OF OREGON.

INTRODUCTION.

The following figures present the results of the raising of XXY Drosophila females in an effort to ascertain, particularly, how increasing maternal age and how differences in the temperature, at which mother flies are maintained, affect the percentage of exceptions to sex linkage.

The first part of the paper deals with the effect of maternal age and here, because of some lack of conformity in the results obtained at different times, the problem has not been solved. However, I feel that the figures should be published for they have been accumulating for three years and I am not, at present, continuing the investigation. The second part of the paper deals with the effect of differences in temperature. Here the different tests which were made check in a fairly satisfactory manner. I am also able to give some figures, in the last part of the paper, which have a bearing upon the genetic variation in exception-producing ability.

EFFECT OF MATERNAL AGE.

The first experiment was carried out during the time I was a graduate student in Genetics at the University of California, the use of successive subcultures being a routine method of rearing flies there and fermented banana the food medium. I undertook, under the direction of the genetic staff, a rather complete repetition of Bridges' 1916 experiments and obtained comparable results in most particulars. However, in a group of females continuously outcrossed and presumably producing a "normal" percentage of exceptions, I obtained 3423 (5.90 per cent.) of

these in 58037 flies; this being somewhat higher than the 4.3 per cent. reported by Bridges.

An inspection of my material, made after most of the data were in, showed that a given female's later subcultures produced, dur-

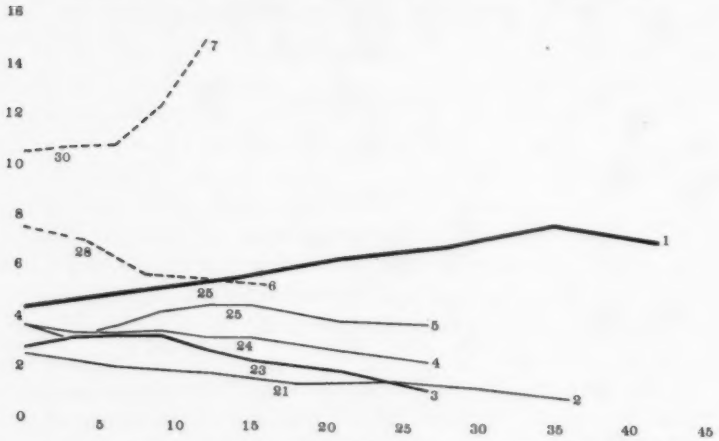

FIG. 1. Curves of smoothed percentages of exceptional young obtained from groups of females kept at different temperatures. Abscissas approximate the age in days of mother females when eggs were ripened. Ordinates are percentages of exceptional young. Curves end with the group number. Under each curve is the temperature in degrees C. Broken lines denote inbred groups. Thick-lined curves are based on more flies.

ing most of her lifetime, almost one per cent. more exceptions per week than her earlier subcultures. The total production of offspring and of exceptions to sex linkage (exc.) of 109 white-eyed females mated with red-eyed males, and changed each week to a new subculture, is given in the totals column of Table I. The curve of these percentages is that of group 1, Fig. 1.

In order to eliminate the possibility of the weighting of the later subcultures by reason of the longer life, or the greater production of exceptions in certain subcultures of a few high-producing females, I excluded, in the figures presented in Table I., the data from all females which produced over 10 per cent. of exceptions or which had any sub-culture failure prior to the one which ended the females production. When the material is divided into groups of females which produced young for the same length of time,

TABLE I.

THE NUMBER OF YOUNG AND THE NUMBER AND PERCENTAGE OF EXCEPTIONS TO SEX LINKAGE IN GROUP 1 WHEN SUBDIVIDED INTO CULTURES PRODUCED BY FEMALES LIVING AN EQUAL LENGTH OF TIME.

Subcultures 7 days apart.

	Subculture													
	A		*B*		*C*		*D*		*E*		*F*		*G*	
	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.
	838	33												
% exc.		3.9												
	1,541	78	1,246	66										
% exc.		5.1		5.3										
	2,304	93	2,356	84	2,254	131								
% exc.		4.0		3.6		5.8								
Smoothed %		3.9		4.5		5.0								
	2,003	88	2,319	121	2,111	146	1,781	119						
% exc.		4.4		5.2		6.9		6.7						
Smoothed %		4.7		5.5		6.2		6.8						
	1,840	68	1,790	96	1,571	108	1,845	114	1,150	100				
% exc.		3.7		5.4		6.9		6.2		8.7				
Smoothed %		4.2		5.2		6.1		7.1		7.6				
	1,519	53	1,376	81	1,246	60	1,229	68	1,310	88	782	71		
% exc.		3.5		5.9		4.8		5.5		6.7		9.0		
Smoothed %		4.2		4.7		5.4		5.7		6.8		8.0		
	1,087	53	859	39	682	43	1,091	53	782	66	838	70	475	21
% exc.		4.9		4.5		6.3		4.9		8.4		8.4		4.4
Smoothed %		4.8		5.1		5.1		6.3		7.0		7.5		6.8
Total	11,132	466	9,946	487	7,864	488	5,946	354	3,242	254	1,620	141	475	21
% exc.		4.2		4.9		6.2		6.0		7.8		8.7		4.4
Smoothed %		4.4		5.0		5.6		6.4		6.9		7.8		7.1

the same sort of "curve of age" is obtained. The production of groups of females, so arranged, being also presented in Table I.

All the females used, in obtaining the above figures, descended from the one original white-eyed female which started the XXY line, so the X chromosomes were presumably constant throughout. Male flies, however, were taken from a number of stocks of flies trapped around Berkeley, or present in the laboratory, so the Y introduced into each generation was not constant. The flies were kept in a large cabinet incubator at 25 degrees C. and counted daily. It is perhaps worth recording that the mother female, of each group of subcultures, was removed from the incubator in the subculture tray during the counting period. This was long enough, at times, to cool the vials down to room temperature.

Before leaving an account of this part of the work I should like to report briefly upon a test of the constitution of 60 regular daughters of XXY females. Twenty-seven of these daughters, when mated with Bar males, produced no exceptions. Thirty-three produced exceptions, but of this number 8 daughters produced just one or two exceptions in large counts of flies, the percentage being, in these cases, consistently in the neighborhood of one third of one per cent.; the total production being 10 exceptions (9 ♀ 1 ♂) in 3,148 flies. If these 8 regular daughters were XX females which produced primary exceptions these latter are not predominantly male as in Safir's results (1920).

Later Experiments.

After some lapse of time during which I was engaged in breeding Peromyscus I returned to the problem of non-disjunction with the idea of checking my results prior to publication, and also of trying out the effect of temperature differences upon the percentage of exceptional offspring. I obtained a stock of flies, through the courtesy of Dr. R. E. Clausen, and after inspecting the progeny of white-eyed females, mated with normal males, picked up an exception-producing strain. The flies used in these latter experiments consisted of a number of white-eyed females from this stock, a number of white-apricot compound females, obtained from a mating of white and apricot, and finally of a

number of white and apricot females obtained by equational non-disjunction from the white-apricot stock, females of which produced XXY daughters pure both for white and for apricot. I could not observe that these allelomorphs, white and apricot, differed from one another in the capacity for exceptional production, in comparable experiments.

The culture methods in this latter part of the work were modified somewhat. Yeast-seeded banana agar was used for food. Females which were producing young were left continuously in the incubator except during the interval when they were changed to a new culture. Subcultures were made up every six days, at 20 or 21 degrees C., and every three days (in group 6, Table III., every four days) at temperatures higher than this. Two Freis electric incubators and one electrically controlled cabinet incubator was used and the temperature checked daily. These machines will fluctuate in temperature, within a degree up or down, but since the routine involved the growth of flies, at each different temperature, over a considerable period of time such fluctuations should cancel out.

Table II. summarizes and Fig. 1 depicts graphically the results obtained when groups of females were kept continuously at certain temperatures each female being transferred to new subculture vials as long as she remained fertile. Except in groups 6 and 7, in which inbreeding was the rule, male parents came from several cultures. In group 6 one inbred stock of wild males was used, in group 7 exceptional brothers.

A comparison of Tables I. and II. and of the curves in Fig. 1 brings to light some very obvious differences in the characteristics of the females in comparable groups. In group 1 the average fertile lifetime, in round numbers, was 28 days. In groups 4 and 5, kept at comparable temperatures, the average fertile lifetime was 16 days. It appears improbable that this difference is an environmental one for banana agar is generally conceded to be a better food medium than fermented banana.

The percentage of exceptional progeny produced by the females in groups 4 and 5 (3.14 and 3.88 per cent.) is considerably below the 5.49 per cent. of exceptions obtained in group 1, and this is not due to the difference in longevity for the group 1 percentage is higher at any comparable age.

TABLE II.

THE NUMBER OF YOUNG AND THE NUMBER AND PERCENTAGE OF EXCEPTIONS TO SEX-LINKAGE OBTAINED FROM GROUPS OF XXV FEMALES KEPT AT DIFFERENT TEMPERATURES.

Gr.	Temp.	Subcultures																	
		A		*B*		*C*		*D*		*E*		*F*		*GH*		I etc.		Total.	
		No.	Exc.	No.	Exc.	No.	Exc.	No.	Exc.	No.	Exc.	No.	Exc.	No.	Exc.	No.	Exc.	No.	Exc.
2.	21°	330	6	571	19	915	11	984	15	740	11	563	9	354	1	—	—	4,457	72
	%		1.8		3.3		1.2		1.5		1.5		1.6		0.3				1.62±.19
	Sm. %		2.5		2.0		1.8		1.4		1.5		1.3		0.9				
3.	23°	3,188	90	2,880	83	2,785	101	1,930	58	1,530	40	1,092	25	658	9	113	1	14176	407
	%		2.8		2.9		3.6		3.0		2.6		2.3		1.4		0.9		2.87±.14
	Sm. %		2.8		3.1		3.2		3.2		2.7		2.3		1.9		1.2		
4.	24°	935	38	1,301	37	954	31	1,156	43	1,131	35	998	28	1,193	42	727	10	8,395	264
	%		4.1		2.8		3.2		3.7		3.1		2.8		3.5		1.4		3.14±.19
	Sm. %		3.6		3.3		3.3		3.4		3.2		3.2		2.7		2.3		
5.	25°	610	25	966	28	844	23	863	44	805	38	579	19	856	44	502	13	6,025	234
	%		4.1		2.9		2.7		5.1		4.7		3.3		5.1		2.6		3.88±.25
	Sm. %		3.6		3.1		3.6		4.2		4.5		4.5		3.9		3.8		
6.	28°	1,239	100	976	60	726	45	639	29	277	17							3,857	251
	%		8.1		6.1		6.2		4.5		6.0								6.51±.40
	Sm. %		7.5		7.0		5.7		5.5		5.3								
7.	30°	1,213	129	740	76	566	64	328	37	127	25							2,974	331
	%		10.6		10.3		11.3		11.3		19.7								11.13±.58
	Sm. %		10.5		10.7		10.8		12.3		14.9								

The curve of age, so obvious in group 1, is not exactly reproduced in any of the later groups although a curve of age, as may be noted in Fig. 1, is usually apparent instead of a randum fluctuation from a model percentage. This curve of age differs at different temperatures, in the figures presented, but the possibility that genetic differences in the females used may be responsible for this cannot be excluded.

A noticeable thing in Table II. is the increase in exception production at higher temperatures. Based on the totals in each group the increase was slow, for a given temperature increment, up to 25 degrees, then more rapid between 25 and 28 degrees and most rapid between 28 and 30 degrees. For these temperatures the curve of increased exception production is comparable to the curve of increased crossing-over of second chromosome genes in Plough's results.

Effect of Temperature Change During the Lifetime of XXY Females.

In order to make tests of this kind females were hatched, and their first eggs ripened at a high temperature and then kept for the remainder of their lifetime at a temperature ten degrees lower. In addition, females were hatched at a relatively high temperature and, after a period at a temperature eight degrees lower, they were returned to the high temperature again. A difference of eight degrees was substituted for ten because too many females died when transferred from a 20 to a 30 degree incubator.

It was found by Plough and Metz that females about twelve hours old have approximately 140 oöcytes in their ovaries. The practice in the experiments here recorded being to empty cultures every 24 hours it can be assumed that the average age at mating, of mother females, was not greater than twelve hours, for younger-appearing females were always chosen for mothers since they are most likely to be virgins. It takes such females six to eight days (this period must differ at different temperatures) to lay the eggs ripened at the temperature at which they were hatched, the lag of layed after ripened eggs continuing all through a female's lifetime.

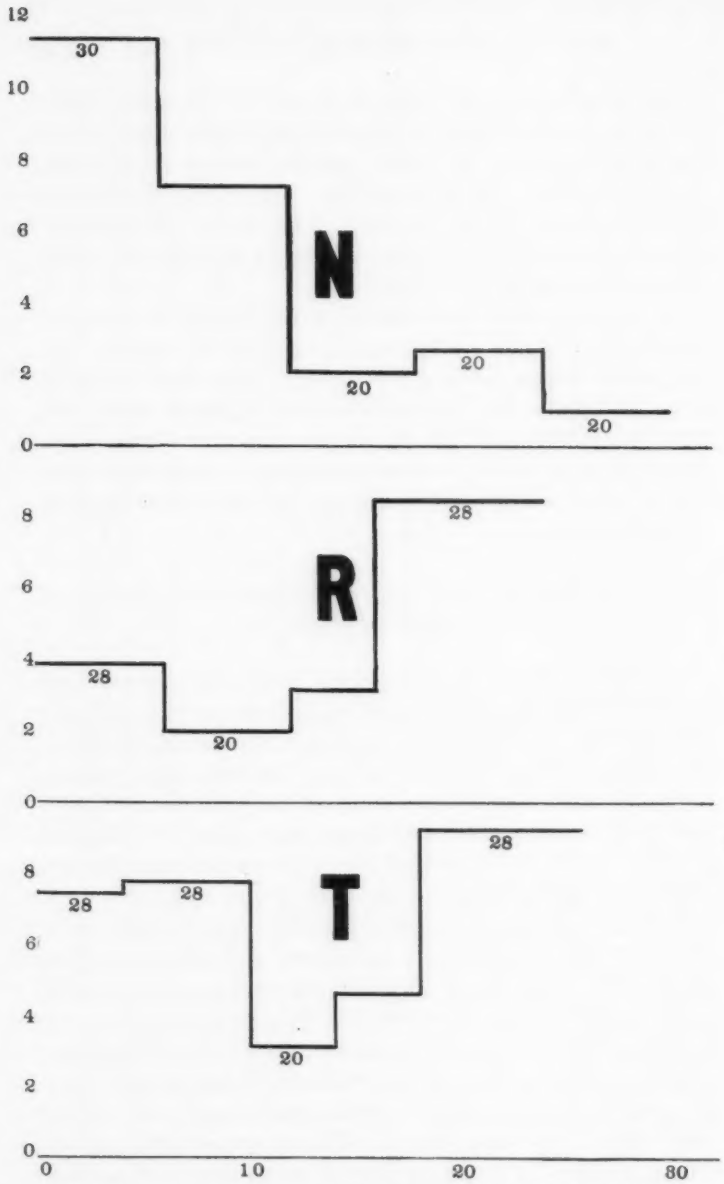

FIG. 2. Curves of three tests of temperature change. Abscissas are days of life of mother females, ordinates the percentages of exceptional young. Figures at intervals, under the curves, are temperatures at which the exception-producing eggs in mother females presumably ripened, intermediate temperature plateaus being left blank.

Table III. and curve *N*, Fig. 2, presents the results obtained when white-apricot compound females, born at 30 degrees C., were mated with normal males and kept at 20 degrees C. Subcultures are six days apart.

TABLE III.

THE NUMBER OF FLIES AND THE NUMBER AND PERCENTAGES OF EXCEPTIONS PRODUCED BY XXY FEMALES BORN AT 30° C. AND THEN MATED AND KEPT AT 20° C.

Temperatures are those at which the eggs, producing the exceptions, are presumed to have ripened.

Subcult.	Temperature.									
	30		30–20		20		20		20	
	A		*B*		*C*		*D*		*E*, *F*	
	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.
	1,751	197	2,005	146	1,931	40	1,023	28	482	5
%		11.2		7.3		2.1		2.7		1.0

It would be expected that the eggs ripened at 30 degrees would be laid in the *A* and in the first part of the *B* subcultures, being followed by eggs ripened at 20 degrees, which would be laid in the latter half of the *B* and in subsequent subcultures. If the percentage of exceptions varies with the temperature at which the eggs are ripened then the *A* subculture should contain the highest, *B* an intermediate and all subsequent subcultures a low percentage of exceptions. It may be seen that this expectation is realized completely.

Table IV. and curve *R*, Fig. 2, presents the results obtained when white-apricot females were born at 28 degrees C., mated with normal males and held for two subcultures (12 days) at 20 degrees C., then returned to 28 degrees C. again.

The expectation of a high *A*, intermediate *B*, low *C*, intermediate *D* and of high subcultures subsequent to *D*, is only partly met. The poor fit in the *A* and *B* subcultures may very well be due to the chance selection of very young mother females. The final cultures are higher than expected.

TABLE IV.

THE NUMBER OF YOUNG AND THE NUMBER AND PERCENTAGES OF EXCEPTIONS PRODUCED BY XXV FEMALES BORN AT 28° C. MATED AND HELD FOR TWO SUBCULTURES (12 DAYS) AT 20° C. AND THEN RETURNED TO 28° C.

Temperatures are those at which eggs are presumed to have ripened.

Subcult.	Temperature.									
	28		28–20		20		20–28		28	
	A		*B*		*C*		*D*		*E, F*	
	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.
	1,084	42	1,146	23	834	15	562	18	434	37
%		3.9		2.0		1.8		3.2		8.5

Table V. and curve *T*, Fig. 2, presents the results obtained when apricot females were born, mated with one inbred stock of wild-type males and held for one subculture (4 days) at 28 degrees C., then kept in a subculture (6 days) at 20 degrees C. and finally returned to 28 degrees C. for all subsequent four-day subcultures.

TABLE V.

THE NUMBER AND PERCENTAGE OF EXCEPTIONAL YOUNG PRODUCED BY XXV FEMALES BORN, MATED, AND KEPT 4 DAYS AT 28° C., THEN KEPT SIX DAYS AT 20° C. AND THEN RETURNED TO 28° C.

Temperatures are those at which eggs are presumed to have matured.

Subcult.	Temperature.									
	28		28		20		20—28		28	
	A		*B*		*C*		*D*		*E, F*	
	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.
	2,342	175	3,825	297	2,108	67	1,033	49	485	45
%		7.5		7.8		3.2		4.7		9.3

This was the most adequate test of the effect of temperature for a fair number of flies was raised, the males used as parents were all of one type, and other females (group 6, Table III.) were held continuously at 28 degrees. The *C* subculture should

be the low one but should not go down to 2 per cent. for the 6 days at 20 degrees would hardly complete the oviposition of all the eggs brought into the culture which were ripened at 28 degrees. The final cultures are again too high and the same cause may be operative here as in Table IV. I suggest an over-reaction to heat after a transfer from a low temperature.

When it is considered that the curve produced by the percentage of exceptional young in different subcultures, which received eggs ripened at different temperatures, is superimposed upon a curve of age which has been shown to be somewhat variable, it may be concluded that the above figures are as close to the requirements for a demonstration of the effect of temperature as could be expected. I think the statement that the percentage of exceptional progeny, produced by XXY females, varies in being higher at high temperatures and lower at low temperatures between 20 and 30 degrees C. is thus quite warranted by the results.

Genetic Differences in Exception Production.

By breeding to reintroduce the maternal Y chromosome, Bridges obtained a "high" line of exception producing females. He suggested that this high production was due to increased heterosynapsis resulting from the peculiar constitution of the introduced Y. Bonnier, using Bridges' high line, showed that the introduction of a new Y is not followed by a return to the "low" percentage of exceptions, and presented evidences to show that a high percentage of exceptions is a matter of the constitution (gene basis) of the two X's.

The following data suggest that neither of these theories will account for all the inherent differences in exception producing ability. I mated 24 matroclinous daughters of a white-eyed XXY female (♀ No. 116), mated with one male, which had herself produced 14.5 per cent. of exceptions in 447 young. These daughters (they are included in Table I. above) produced an average of 6.2 per cent. of exceptions in 11,692 young, the percentage of individual cultures being from 2.9 per cent. in one culture of 820 young to 10 per cent. in another culture of 600 young. This difference between 2.9 and 10 per cent., is five times its probable

error[1] although these daughters all had, theoretically at least, equivalent sex chromosomes. Here an apparent segregation of exception-producing ability is manifest.

It can readily be demonstrated in another way that this variability is not just a matter of random sampling, for if the percentage of exceptions produced during the first part of a female's lifetime is compared with that produced during a later part of her lifetime, positive correlation may be observed. I made such calculations within groups of females which had produced a sequence of complete subcultures at 23, 26 and 30 degrees (groups 3, 1, and 5 already tabulated) and obtained the following correlation coefficients: $+.56 \pm .12$, $+.39 \pm .07$ and $+.70 \pm .11$. This suggests that the ability to produce a certain percentage of exceptions, tends, like any other measurable character, to stay within the limits proscribed by genetic constitution of the individual in question. That being the case one would expect parent-offspring correlation, in exception-producing ability, of about the same magnitude as that which has been found for other characters. I had one series in which I could test this point, for in group 1 there were 60 daughters which had produced 300 or more young which had mothers with an equal productivity. The mother-daughter correlation in exception production was here $+.37 \pm .07$.

I selected a few generations of flies for increased exception-production by choosing the daughters of my higher producing females and mating these daughters with their exceptional brothers. In the first selected generation I obtained 6.5 per cent. of exceptions in 4,493 flies, in the second generation 8.4 per cent. of exceptions in 4,414 flies, and in the third generation 14.6 per cent. of exceptions in 1,772 flies. Exceptional females from average exception-producing mothers which had been similarly mated with their exceptional brothers produced 5.6 per cent. of exceptions in 5,674 flies, so inbreeding alone did not increase the per cent. of exceptions. An inspection of the pedigrees showed the selected high line to altogether come from one high-producing

[1] When the probable errors are obtained by the formula $\pm\sqrt{\frac{p \cdot q}{N}}$, where p = the percentage, $q = 100 - p$ and N = the number of observations upon which p is based.

female. After one generation produced by outcrossing to non-related stock, two females from this line still produced 14.1 per cent. of exceptions in 546 flies.

I also repeated Bridges' scheme of mating to reintroduce the maternal Y chromosome from a high-producing female by mating her daughters with their exceptional uncles. In two generations of this line I obtained 15.5 per cent. of exceptions in 2,072 flies, a figure comparable, since sister females were used in the two cases, to the third and one later generation of the high selection tabulated above. Either of these methods of inbreeding would tend to concentrate factors favorable to exception production in a given line of flies and these need not be intrinsic characteristics of the sex chromosomes.

Conclusions.

The data presented above together with those of previous investigations show that the percentage of exceptions to sex linkage may be affected by a number of variables which, in order of importance, are: the genetic constitution of the female, the temperature at which eggs are ripened, and maternal age.

With regard to genetic constitution, XX females produce well under 1 per cent. of primary exceptions. XXY females may produce from 1 to over 20 per cent. Our knowledge of the causes of this latter variability does not appear to be complete. Bonnier's outcrossing experiments appear to remove the probability that the constitution of the Y chromosome is responsible for Bridges' high eosin line and although Bonnier was not able to exclude the possibility that autosomal genes were implicated, his experiments pointed to an exclusively X chromosomal effect in the production of different percentages of exceptions. It is the rule to have a constant pair of X chromosomes in all lines ot secondary non-disjunction, except as the X's may interchange material with each other or with the Y, and yet genetic variability is still present. The only inference that is possible is that the percentage of exceptions may be affected directly by interaction of the sex chromosomes themselves and indirectly by autosomal

genes.[2] This is what has been found to be true in cross-over percentages.

The direct effect of temperature upon the percentage of exceptions appeared in all the tests I made. The results of temperature differences upon protoplasm in modifying physiological activity are so well known that some temperature effect would be an à priori expectation in secondary non-disjunction. Although my data suggest that the temperature effect increased as 30 degrees C. was approached, I cannot exclude the possibility that this was due to genetic differences in the groups of females kept at these different temperatures.

Maternal age appears to affect the percentage of exceptional young to some degree but apparently by interacting with other variables for different age curves were found in different groups and at different temperatures.

The inference that autosomal genes, temperature and maternal age all affect the allocation of the sex chromosomes, in XXY females, to gamete-forming cells, follows the conclusions reached above.

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[2] This would explain why a high-producing female may or may not establish a high exception-producing line.

OXYGEN CONSUMPTION OF INSECT EGGS.[1]

ROY MELVIN,

IOWA STATE COLLEGE.

Although insect eggs present unique material for studies in metabolism the literature contains comparatively few reports in this interesting field of insect physiology. Bodine (1) has presented data from a study of eggs of Orthoptera which show the velocity of development to increase in direct proportion to increase in temperature within the normal limits of development. This is in accordance with other findings on the effects of temperature on poikilothermos species. This author shows that it is possible to calculate the time of hatching of eggs if previous temperature history is known. Such knowledge of insect pests may lend itself to practical application.

Fink (2) has conducted studies which lead him to conclude that the formative period in the development of eggs of certain insects is dependent upon whether they are deposited upon foliage or in the soil. Data to be presented in this paper tend to disprove the above explanation. For further references to literature in this field see the papers of Bodine and Fink cited above.

Thanks are due Doctor Erma Smith, Professor of Physiology, and other members of the Zoölogy and Entomology staff at Iowa State College, for many helpful suggestions and encouragement throughout the course of this work.

PURPOSE.

The purpose of this paper is to present briefly a preliminary report of a study of the oxygen consumption during embryonic development of certain insects.

[1] Contribution from the Department of Zoölogy and Entomology, Iowa State College, Ames, Iowa.

Methods and Material.

Bodine's modification of Krogh's manometer was used for determining the oxygen intake. Constant temperatures were maintained by use of a Freas electric water bath. The data presented were determined from the eggs of the following insects: Squash bug, *Anasa tristis* De G.; Luna moth, *Tropæa luna* L.; Cecropia moth, *Samia cecropia* L.; and Smartweed borer, *Pyrausta ainsliei* Hein.

The O_2 consumption is expressed in millograms of O_2 per gram live weight (exclusive of shell) per hour. Apparently previous workers have not taken into consideration the weight of the shell. If the weight of the shell be deducted, as it evidently should be, the O_2 consumption curve will be raised from 10 to 30 per cent.

The per cent. of shell at the beginning of incubation for several species of insects was found to be as follows: *A. tristis*, 29.2; *S. cecropia*, 22; *T. luna*, 23.3; and *P. ainsliei*, 31.

Assuming the weight of the egg shell to remain constant throughout the incubation period, the percentage of shell varies directly with changes in the weight of the egg. It is thus evident that changes in the weight of the egg will alter the type of curve representing O_2 consumption. For this reason the weight was determined just prior to each gas determination and calculations made accordingly.

Determinations were made on egg masses as soon as they were deposited and every 12 or 24 hours thereafter, depending on the length of the incubation period, until hatched. Calculations were made according to the formula of Krogh (3).

Temperature.

The effects of temperature upon biological processes are too numerous and too well known to warrant detailed discussion. Numerous investigators have studied the effect of temperature upon the length of the incubation period, but few reports have been found dealing with the effect of temperature upon the rate of metabolism as determined by the oxygen consumption. With this in mind experiments were undertaken to determine the effects of temperature upon embryonic development of insects.

TABLE I.

SHOWING SUMMARY OF DATA ON EFFECTS OF TEMPERATURE ON O_2 CONSUMPTION DURING EMBRYONIC DEVELOPMENTS OF *S. cecropia*.

Temp.	Manner of Calculation.	Total No. of		Milligrams of O_2 Used per gram of Live Weight per Hour by Days.								
		Masses.	Eggs.	1	2	3	4	5	6	7	8	9
28	Exclusive of shell	11	534	.345	.359	.500	.615	.632	.684	.860	1.262	1.817
28	Inclusive of shell	11	534	.260	.320	.381	.480	.500	.520	.672	.985	1.420
34	Exclusive of shell	4	213	.347	.415	.550	.653	1.150	1.660	2.560	3.460	
34	Inclusive of shell	4	213	.287	.321	.420	.525	.900	1.302	2.000	2.710	

TABLE II.

SHOWS THE MILLIGRAMS OF O_2 CONSUMED PER GRAM OF LIVE WEIGHT (EXCLUSIVE OF SHELL) PER HOUR.

Name of Insect.	Total No. of										
	Egg Masses.	Eggs.	1	2	3	4	5	6	7	8	9
A. tristis	8	606	.383	.580	.890	.985	1.262	2.000	2.800		
S. cecropia	11	534	.345	.350	.500	.615	.632	.684	.860	1.262	1.817
T. luna	9	501	.397	.410	.421	.650	1.270	2.000	2.800		
P. ainsliei	4	630	1.400	1.500	1.800	2.200	3.000	6.000			

The same eggs were used to make the determinations at both temperatures. After sufficient acclimatization, two to five hours, depending on the size of the animal chamber, the manometer was closed and at the end of two hours the reading was made. In like manner the reading for the next temperature was made and the eggs returned to the incubator at 28 degrees until the next day.

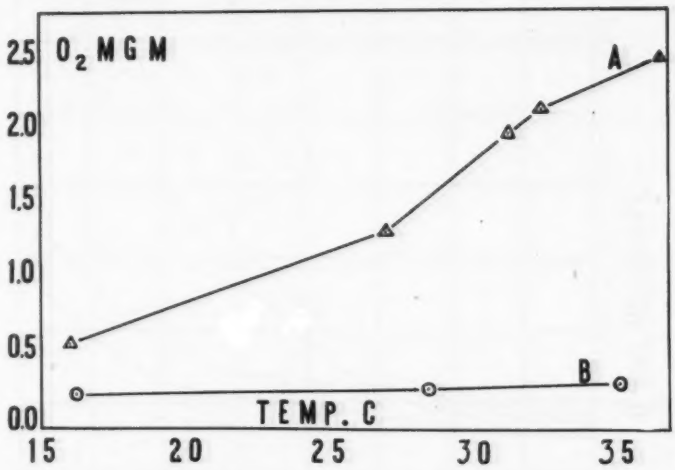

FIG. 1. Showing the effects of temperature on the O_2 consumption of *T. luna* eggs. (*A*) last day of incubation; (*B*) first day of incubation.

The results of these experiments are shown graphically in Fig. 1.

It is observed from Fig. 1 that the effects of temperature on O_2 consumption are very slight during the first day of incubation and very pronounced during the last day. In order to determine the effect of temperature on O_2 consumption during the entire incubation period daily determinations were made on two series of *S. cecropia* eggs at 28 and 34 degrees C. respectively. These results are summarized in Table I. and shown graphically in Fig. 2.

For comparative purposes the eggs of four species of insects were run at 28 degrees C. and the rate of O_2 consumption thus determined is shown graphically in Fig. 3 and summarized in Table II.

Formative Period.

Upon examination of Figs. 2 and 3 we note that during the early part of the incubation period temperature has very little stimulation on the O_2 consumption but as the incubation period

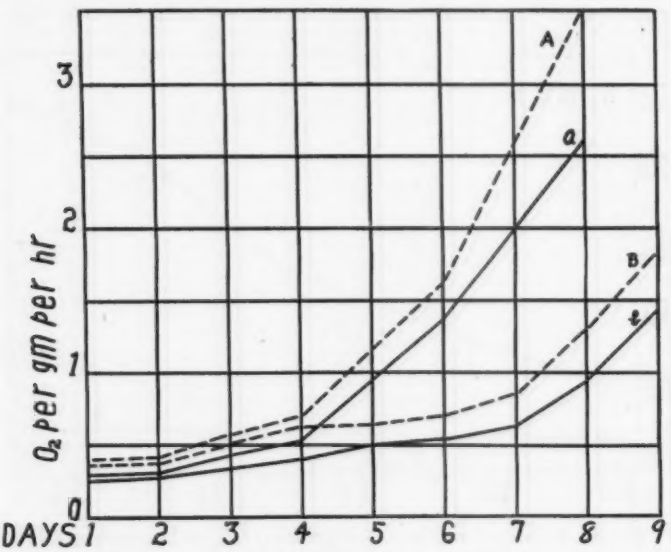

Fig. 2. Shows the effects of temperature on O_2 consumption during the entire incubation period of *S. cecropia*. (*A*) exclusive of shell 34° C., (*a*) same as above but including shell; (*B*) exclusive of shell 28° C., (*b*) same as above, but including shell.

progresses its effects become pronounced. This is in accord with and substantiates the existing theory which states *that during early embryonic development there is a formative period during which metabolic activity is comparatively low and only influenced slightly by environmental changes.* Mention has been made, above, of the explanation offered by Fink for the variation in the length of this formative period among different species of insects. In the case of *S. cecropia* and *T. luna,* both species laying eggs on foliage, the formative period is somewhat lengthened. This is contrary to Fink's explanation. Data presented in this paper shows the length of the incubation period to be a greater

factor in determining the length of the formative period than the type of place where the eggs chance to be deposited. Fig. 3

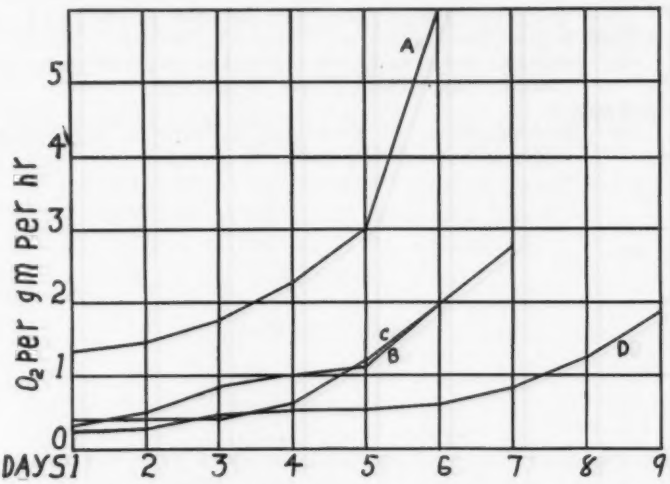

FIG. 3. O_2 consumption of eggs of (*A*) *P. ainsliei*, (*B*) *A. tristis*, (*C*) *T. luna*, and (*D*) *S. cecropia*.

bears out this explanation. A study of Fink's curves will show that they too substantiate the explanation here offered.

SUMMARY.

From a preliminary study of the factors accompanying and influencing metabolism as determined by the O_2 consumption during embryonic development made on four species of insects the following conclusions are drawn:

I. The weight of the egg shell is an important factor and should be taken into consideration.

II. The effects of temperature are not as pronounced during the formative period as during the period of late incubation.

III. The explanation offered for the variation in the length of the formative period is the length of the incubation period and not the place where the eggs chance to be laid as has been suggested.

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